昭和 32 年 7 月 9 日 第三種 郵便物 認可
第 45 巻 第 11 号 昭和 33 年 11 月 25 日発行
(毎月 1 回 25 日発行)
The Journal of Biochemistry, published monthly by the Japanese Biochemical Society, Tokyo, Japan.
Vol. 45, No. 11, November 25, 1958

Vol. 45, No. 11 **November, 1958**

THE JOURNAL OF BIOCHEMISTRY

EDITED FOR THE JAPANESE BIOCHEMICAL SOCIETY

PUBLISHED MONTHLY

BY

THE JAPANESE BIOCHEMICAL SOCIETY

Tokyo University, Tokyo

AGENCIES

Charles E. Tuttle
Rutlant, Vt.
U.S.A.

Maruzen Co. Ltd.
Tokyo
Japan

Santo Vanasio
Köln
Germany

PRINTED IN JAPAN

THE JOURNAL OF BIOCHEMISTRY was founded in 1922 by Prof. emeritus Dr. S. Kakiuchi and had been edited by him to volume 36 of the year of 1944. After the discontinuation during the War, this JOURNAL has been restarted by The Japanese Biochemical Society, following the serial number from volume 37 of the year of 1950.

This JOURNAL is collecting the original investigations on the subject in this country. It is published monthly, one volume per year.

Note to Contributors

All manuscripts should be carefully typewritten, triple-spaced, in English, French or German, and addressed to *The Editor, The Journal of Biochemistry, The Japanese Biochemical Society, c/o Departments of Biochemistry, Faculty of Medicine, Tokyo University, Bunkyo-ku, Tokyo, Japan.*

Authors receive 50 reprints of their papers as a rule at cost; additional copies can be prepared by the order on cost accounts. All orders for reprints should be markedly noted on the article paper or in a sheet of paper attached to it. On submitting the paper, it is preferable to attach the non-traced charts, besides original drawings, for circulation to Editors.

On request from authors to contribute to JOURNAL, the *Directions for preparation of manuscripts*, including styles of abbreviations of symbols, terms, units, and periodicals will be sent immediately.

Subscription

Subscription Price. ¥3,600 (U. S. $10.00) per volume, postage inclusive. Back issues are available for sale, from volume 1 up-to-date at ¥3,600 (U.S. $10.00) per volume, postage inclusive.

Remittances should be made preferably by check or draft payable to *The Japanese Biochemical Society, c/o Department of Biochemistry, Faculty of Medicine, Tokyo University, Bunkyo-ku, Tokyo, Japan.*

Foreign subscription is treated by the agencies of *Charles E. Tuttle, Co.*, Rutland, Vermont, U.S.A., *Santo Vanasio*, Köln, Germany, and *Maruzen Co. Ltd.*, Chuo-ku, Tokyo, Japan.

The Journal of Biochemistry, Vol. 45, No. 11, 1958

STUDIES ON CYTOCHROME A

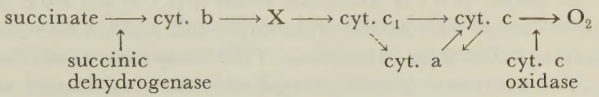
I. EXTRACTION, PURIFICATION AND SOME PROPERTIES OF CYTOCHROME A

By KAZUO OKUNUKI, ICHIRO SEKUZU, TAKASHI YONETANI, AND SHIGEKI TAKEMORI

(From the Department of Biology, Faculty of Science, University of Osaka, Osaka)

(Received for publication, February 7, 1958)

In 1940, Yakushiji and one of the present authors (K.O.) discovered a new cytochrome component in heart muscle and named it cytochrome c_1 (*1*). Through the development of the purificatin procedure for this component, they also succeeded in extracting and purifying cytochrome a from heart by using sodium deoxycholate (*2*, *3*). This cytochrome a preparation had no cytochrome oxidase activity, but could combine with cyanide and carbon monoxide. The preparation was reduced rapidly by reduced cytochrome c and slowly by cytochrome c_1. From these and other (*4*, *5*) observations, the pathway of electron transport in the succinic oxidase system of heart muscle was represented by them as follows;



Based on the classical pictures of Keilin (*6*, *7*) and Warburg (*8*, *9*), however, Slater (*10*, *11*) and Chance (*12*) have laid great emphasis upon an entity, cytochrome a_3, which was distinguishable from cytochrome a by its behavior towards cyanide and carbon monoxide. Their theories on cytochromes a and a_3 were supported by the observations of Straub (*13*), Kiese *et al.* (*14*), Smith and Stotz (*15*), and Stotz *et al.* (*16*), who also used sodium cholate or deoxycholate for purification of these components.

Thus our efforts were mainly directed to the settlement of discrepancies between the scheme of the electron transport system proposed by us and by other workers. This and following papers will present the properties of the cytochrome a preparation purified by our improved procedure, and will show the relation between cytochrome a and cytochrome oxidase. The present paper deals with details of the extraction and purification methods and gives some properties of the cytochrome a preparation obtained.

MATERIALS AND METHODS

Cholic acid was crystallized from bovine bile by the method of Gatterman (*17*)

and recrystallized twice from ethanol. Crystalline cytochrome c was prepared from beef heart according to the method of Hagihara *et al.* (*18*). This crystalline preparation was dissolved in 0.1 *M* phosphate buffer (pH 7.0), and after a small amount of ferricyanide had been added it was dialyzed against the same buffer for 4 hours. Reduced cytochrome c was prepared according to the method of Theorell (*19*) by means of Pd-H_2. The concentration of cytochrome c was calculated from the extinction coefficient of reduced form at 550 mμ, assuming that this has a value of 27.7×10^3 $M^{-1} \cdot cm^{-1}$ (*20*). Alumina Cγ gel was prepared by the method of Willstäter and Kraut (*21*).

Spectrophotometric determination was made using a Shimadzu spectrophotometer type QB-50 with cells of 1 cm. light path. Cytochrome oxidase activity was determined spectrophotometrically by measuring the rate of decrease in optical density at 550 mμ of reduced cytochrome c according to the method of Smith (*22*). Protein concentration was represented as nitrogen precipitated by 0.4 *N* trichoroacetic acid. The nitrogen content was measured by the micro-Kjeldahl method. Iron and copper concentrations were measure with o-phenanthroline and sodium diethy dithiocarbamate, respectively,using a modified method of Sandell (*23*). Gram solid ammonium sulfate to be added to a solution to give a certain saturation were calculated from the Table of Green and Hughes (*24*).

Isolation and Purification of Cytochrome a

1. Preparation of Heart Muscle Particle Suspension (Green Brei)—A fresh heart, previously trimmed of visible fat and fibrous tissues, is passed once through a meat grinder. The minced heart is washed for about 30 minutes, with occasional stirring first, with about 10 volumes of cold tap water at under 10°, then twice with about 2 volumes of cold 0.02 *M* disodium phosphate and finally twice with cold tap water. The washed mince is collected by straining through a cloth. One kg. of the mince is mechanically ground in a mortar for 20 minutes with 300 ml. of 0.1 *M* phosphate buffer (pH 7.4) and 500 g. of quartz sand. The homogenate is diluted with 500 ml. of the buffer and distilled water to 4,000 ml. It is then centrifuged at $2,000 \times g$ for 15 minutes. The cluody supernatant fluid is decanted off and saved. The precipitate is homogenized and centrifuged again as before. The supernatants of the first and second centrifugations are combined. Usually 2,500 to 3,000 ml. of the fluid is obtained. The pH of the cold supernatant fluid (at under 5°) is reduced to 5.6 by the addition of cold 1 *M* acetic acid, and the mixture is immediately centrifuged at $3,000 \times g$ for 20 minutes. The clear red supernatant fluid is discarded, and the precipitate is washed with cold distilled water and centrifuged. The precipitate is suspended in about an equal volume of 0.1 *M* phosphate buffer, pH 7.4, and the pH adjusted to 7,4 with 1 *N* NaOH (the final volume should be about 450 ml.).

2. Extraction of Cytochrome a—To 450 ml. of Green Brei which has been kept at 5° overnight, 112.5 ml. of 10 per cent sodium cholate (pH 7.4) and then 81 g. of ammonium sulfate are added and the pH is adjusted to 7.5 with 4 *N* NH_4OH. The mixture is allowed to stand at 10° for 2 hours. Thirty minutes before centrifugation, the mixture is made up to 0.35 saturation by the further addition of 36.5 g. of ammonium sulfate. The precipitate is removed by centrifugation ($9,000 \times g$, 20 minutes) and clear (sometimes slightly turbid reddish supernatant fluid (Fraction S_1) which contains cytochrome a, b, c, and c_1, is decanted and made to 0.55 saturation with ammonium sulfate. Usually 450ml. of the extract is obtained, so 58 g. of ammonium sulfate is needed to precipitate the cytochromes. After adjusting the pH to 7.5 and placing in an ice box for 30 minutes, the suspension is centrifuged as before. The precipitate contains cytochromes a, b, and c_1, while the supernatant fluid has only a c-component of cytochrome. The precipitate is dissolved in 100 ml. of 0.1 *M* phosphate buffer (pH 7.4) containing 0.5 per cent sodium

cholate and saturated to 0.25 with ammonium sulfate (Fraction S_2).

3. Removal of Cytochromes b and c_1—The above solution is stored overnight in an ice box. During this time, most of the cytochromes b and c_1 become insoluble and precipitate. After centrifugation (7,000 × *g*, 15 minutes) the supernatant fluid is made up to 0.35 saturation with saturated ammonium sulfate solution buffered to pH 7.5. Cytochrome a and the rest of the cytochromes b and c_1 are precipitated and only a little cytochrome c_1 remains in the supernatant. The precipitate is dissolved in 50 ml. of the above 0.1 *M* phosphate buffer (containing 0.25 saturation of ammonium sulfate and 0.5 per cent sodium cholate) and by addition of more ammonium sulfate the fraction between 0.25 and 0.35 saturation is obtained (Fraction S_3). This salting out is repeated several times and finally the solution is stored at 0.25 saturation at 5° for 4 days. By these treatments, a clear deep reddish, concentrated solution of cytochrome a is obtained (Fraction S_4).

4. Alumina C_γ Oel Adsorption—By the treatment described in the foregoing section, cytochrome a is purified and no other components are observed spectrophotometrically, although there is sometimes a small shoulder at about 430 mμ. To purify cytochrome a further, the solution is dialyzed against 2 liters of 0.02 *M* Na_2HPO_4 for 4 hours and then 2 g. of alumina C_γ gel well washed with distilled water is added. After keeping for 30 minutes in an ice box, the gel, which adsorbs cytochrome a completely, is collected by centrifugation and washed with 0.02 *M* Na_2HPO_4 containing 0.5 per cent sodium cholate, and then with 0.05 *M* Na_2HPO_4 containing 0.5 per cent sodium cholate. Cytochrome a can be eluted from the gel by 0.07 *M* Na_2HPO_4 containing 0.5 per cent sodium cholate. The eluate is further purified by precipitation with ammonium sulfate. In this way purified cytochrome a is obtained.

RESULTS

Absorption Spectra—The absorption spectrum of the purified cytochrome a shows three distinct peaks at 280 mμ, 424 mμ, and 600 mμ in the oxidized form, and the bands in visible region correspond to the α and γ bands, respectively. After reduction with sodium dithionite, the α and γ bands shift to 605 mμ and 444 mμ, as illustrated in Fig. 1. The ratio of the optical density at 444 mμ to that at 605 mμ (E_{444}/E_{605}) is usually constant in our various preparations of cytochrome a and the value is illustrated in Table I. In the crude preparations which are free from other components, a small shoulder in the region of the γ-band can be seen. This shoulder disappears after further purification with alumina C_γ gel. The band corresponding to the β-band found by Ball *et al.* (*25*) is not observed in our preparation. These results show that all other cytochrome components have been completely removed from the preparation.

Analysis of Heme—After the addition of pyridine to the dithionite-reduced cytochrome a solution, the pH was adjusted to 10 with 1 *N* NaOH, α the and γ bands then shift to 585 and 430 mμ, respectively, as illustrated in Fig. 2. The heme, separated from the protein moiety with acid acetone (1 ml. of 20 per cent HCl plus 100 ml. of acetone) and purified by a chromatography on an alumina column, when treated with pyridine at pH 10 shows the same absorption spectrum observed with cytochrome a treated with pyridine. These results indicate that the heme of cytochrome a belongs to the

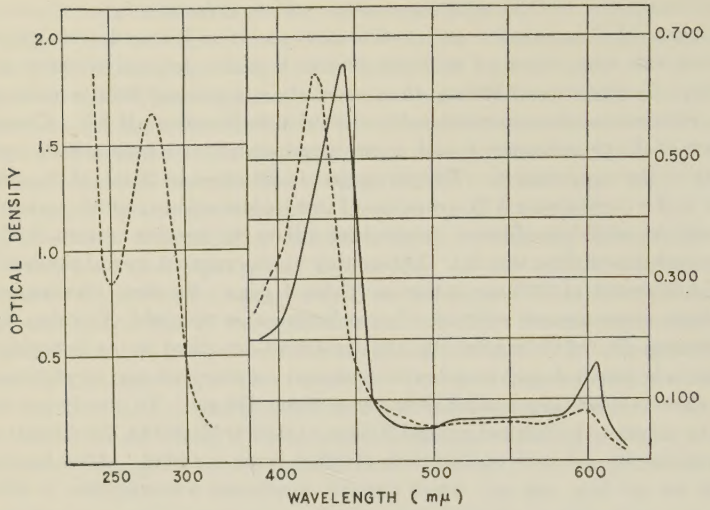


FIG. 1. Absorption spectra of oxidized and reduced cytochrome a. Optical density readings were taken at 5 mμ intervals over the entire wave-length range and at 2 mμ intervals at each absorption maximum or minimum. The spectra were read in 0.1 *M* phosphate buffer (pH 7.4) containing 1.0 per cent sodium cholate; for reduction a small amount of sodium dithionite and for oxidation a small amount of potassium ferricyanide were added. The control cuvette contained the same buffer containing cholate and the same amount of reduciung or oxidizing agent. Solid line, reduced form; Dotted line, oxidized form.

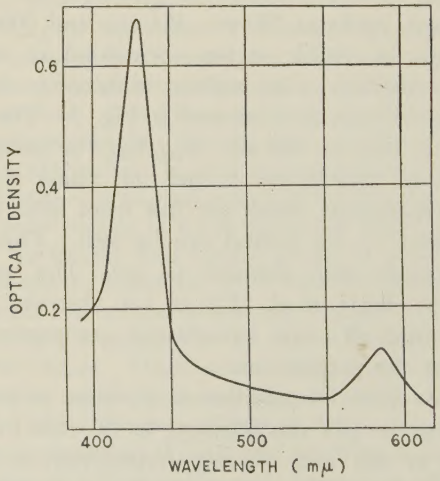


FIG. 2. Absorption spectrum of pyridine hemochromogen of cytochrome a.

dichromatic heme.

Electrophoretic and Ultracentrifugal Analyses—Experiments are carried out

at pH 7.6 and in 0.1 *M* phosphate buffer containing 0.5 per cent sodium cholate. On electrophoretic analysis, the preparation shows a single sharp peak of colored protein, and some broad peaks of uncolored protein which moved more quickly. By ultracentrifugal analysis, two main peaks are found, one of which is colored and the other is uncolored and smaller. From these results, it is presumed that the purity of cytochrome a preparation is about 70 per cent.

TABLE I

Extinction Ratio and Contents of Iron and Copper of Cytochrome a Preparation

Optical density			Extinction ratio			Fe	Cu
280 mμ (Oxidized)	444 mμ (Reduced)	605 mμ	$\frac{E_{280}}{E_{444}}$	$\frac{E_{280}}{E_{605}}$	$\frac{E_{444}}{E_{605}}$	(10^{-2} μM per *mg.* N)	
Per *mg.* nitrogen			2.5	10.3	4.1	6.3	28.2
13.44	5.34	1.30				Cu/Fe	
per μM Fe						4.5	
	84.63	20.64					

TABLE II

Effect of Dialysis on the Copper Content of Cytochrome a

Four ml. samples of cytochrome a were dialyzed against 250 ml. of a solution of each chelating agent (pH 7.5) for 35 hours at 5°. The copper content is given as a difference between the copper in cytochrome a and that in the equal volume of surrounding solution.

Dialysis solution	Copper content (μg./*mg.* N)	Recovery (%)
(A) 0.5 *M* Na_2HPO_4 +0.5% Na-cholate	18.0	100
(B) (A)+0.05 *M* KCN	3.5	19.5
(C) (A)+0.05 *M* 8-hydroxyquinoline	4.1	22.8
(D) (A)+0.02 *M* Diethyl dithiocarbamate	6.9	38.7

Analyses of the Iron and Copper Contents—Results of analyses of iron and copper are summarized in Table I. The ratio of the copper to iron content is 4.5. From the result of hemin analysis using the acid-acetone method, more than 90 per cent of the total iron is accounted for as heme-iron, while more than 90 per cent of the total copper is recovered in the non-heme moiety of the preparation.

Most of the copper can be released from the protein moiety by dialysis for 35 hours against a solution containing a metal chelating agent such as sodium diethyl dithiocarbamate, 8-hydroxyquinoline, and potassium cyanide, as illustrated in Table II. The preparation shows no peak in its absorption spectrum which could be attributed to the combination of copper with the protein moiety, such as is observed in the copper enzyme butyl-Co-A dehydrogenase. That cytochrome a contains both iron and copper is a specific property which is not observed in any other cytochrome. This result agrees with the report of Green (*26*).

Effect of Oxidizing and Reducing Agents—Cytochrome a at between pH 7 to 8 is reduced by sodium dithionite, *p*-phenylenediamine or reduced cytochrome c. Other reducing agents such as hydroquinone, ascorbic acid, sodium borohydride, hardly reduce it in the absence of a small amount of cytochrome c.

Reduced cytochrome a is rapidly oxidized by potassium ferricyanide. The reaction product with oxygen gives a different absorption spectrum from that of this oxidized form, and this will be described in detail in the following papers.

Cytochrome Oxidase Activity—Table III illustrates the cytochrome oxidase activity of the preparation during the purification process. As can be seen from the data, the final preparation retains only a weak activity, its maximum activity corresponding to one thirteenth of the value observed in Fraction S_2. The activity is not recovered in any other than the cytochrome a fraction and the lost activity is not regained by the addition of any other fraction to the cytochrome a preparation. Furthermore, it was clearly

TABLE III

Cytochrome Oxidase Activity During Purification of Cytochrome a

Fraction	Cytochrome components	Total volume (*ml.*)	Nitrogen (*mg./ml.*)	Activity ($sec.^{-1}$/*mg.* N)	Total activity ($sec.^{-1}$)	Activity (%)	Copper (*μg.*/*mg.* N)	Activity ($sec.^{-1}$/*μg.* Cu)
Particle preparation	$abcc_1$	400	11.45	0.57	2648	100	1.8	0.32
S_1	$abcc_1$	430	5.78	1.02	2545	96	2.6	0.38
S_2	abc_1	110	3.72	4.63	1892	71	4.3	1.09
S_3	ab_1	45	3.61	1.25	202	8	7.7	0.16
S_4	a	14.6	2.42	0.19	6.9	0.3	14.4	0.013

demonstrated that the copper content of the fractions are not proportional to their activity. The relation between the activity and iron content will be reported in the following papers.

Stability—The preparation is very stable in 0.1 *M* phosphate buffer containing 0.5 per cent sodium cholate, and its stability is increased by the addition of ammonium sulfate to the solution.

DISCUSSION

The solubilization of cytochrome a from heart muscles particles can b effected in the presence of both cholate and ammonium sulfate, but th cytochrome not by the incubation with cholate alone. After extracting th cytochrome, however, cholate is necessary to keep it in the soluble state By spectral analysis, the purified pigment appears to be homogeneous with respect to its heme component. However, both by electrophoresis and by ultracentrifugation more than one component are found. A β-band canno be observed in the purified preparation.

It is of special interest that cytochrome a contains not only iron bu also copper. In the known copper enzymes, such as tyrosinase, phenolase and butyl-Co-A dehydrogenase, copper is an important component of th prosthetic group, but is released from the protein moiety by dialysis against potassium cyanide. Since the copper in cytochrome a is also partially removed by dialysis against metal chelating agents, the bound from of copper in cytochrome a must be unlike that of iron in hemoproteins, but similar to the copper in these copper enzymes. The role of copper in the electron transfer system is still obscure. Recently Green (*26*) reported that copper and "hemoprotein a" in his election transfer particle are almost all in the green particles. On the other hand, from dietary experiments, many investigators (*27—31*) observed that copper-deficient tissues had a low cytochrome oxidase activity and a decreased content of hemin a. The role of copper in cytochrome a should be clarified by further investigation. In this connection, the fact that the solubilized cytochrome a preparation has a weak cytochrome oxidase activity as compared with the particle preparation is of interest. This observation will also be reported in detail in the following papers.

SUMMARY

1. A purified preparation of cytochrome a is obtained from beef heart muscle by extraction with sodium cholate and ammonium sulfate, fractionation with ammonium sulfate, and adsorption on alumina C_γ gel.

2. As judged from its absorption spectrum and ultracentrifugal and electrophoretic behaviors, this preparation appears to contain only one heme component.

3. The preparation contains not only iron but also copper. More than 90 per cent of iron is present as heme-iron and more than 90 per cent of copper is in the non-heme moiety.

4. The preparation has cytochrome oxidase activity, but its activity is very low compared with the activity found at earlier stages of purification.

We are most grateful to Dr. K. Takeda, director of the Research Laboratory of Shionogi & Co., Ltd., Osaka, for his gift of cholic acid.

REFERENCES

(*1*) Yakushiji, E., and Okunuki, K., *Proc. Imp. Acad.*, **16**, 299 (1940)
(*2*) Yakushiji, E., and Okunuki, K., *Proc. Imp. Acad.*, **17**, 38 (1941)
(*3*) Okunuki, K., and Yakushiji, E., *Proc. Imp. Acad.*, **17**, 263 (1941)
(*4*) Okunuki, K., and Yakushiji, E., *Proc. Japan Acad.*, **24**, (10) 12 (1948)
(*5*) Okunuki, K., and Yakushiji, E., *Proc. Imp. Acad.*, **16**, 144 (1940)
(*6*) Keilin, D., and Hartree, E.F., *Proc. Roy. Soc. Lond.*, B **127**, 167 (1939)
(*7*) Keilin, D., and Slater, E.C., *Brit. Med. Bull.*, **9**, 89 (1953)
(*8*) Warburg, O., and Negelein, E., *Biochem. Z.*, **193**, 339, (1928)
(*9*) Warburg, O., *Z. Ang. Chem.*, **45**, 1 (1932)
(*10*) Slater, E.C., *Biochem. J.*, **46**, 484 (1950)
(*11*) Slater, E.C., *Biochem. J.*, **46**, 499 (1950)
(*12*) Chance, B., and Williams, G.R., *J. Biol. Chem.*, **209**, 819 (1954)
(*13*) Straub, F.B., *Z. Physiol. Chem.*, **268**, 227 (1941)
(*14*) Dannenberg, H., and Kiese, M., *Biochem. Z.*, **322**, 395 (1952)
(*15*) Smith, L., and Stotz, E., *J. Biol. Chem.*, **209**, 819 (1954)
(*16*) Stotz, E., Morrison, M., and Marinetti, G., in " Enzymes: Units of Biological Structure and Function ", Academic Press Inc. Publishers, NewYork. p. 401 (1956)
(*17*) Gattermann, L., in " Die Praxis des organischen Chemikers ", Walter de Gruyter & Co., Berlin, p. 407 (1925)
(*18*) Hagihara, B., Morikawa, I., Sekuzu, I., Horio, T., and Okunuki, K., *Nature*, **178**, 629 (1956)
(*19*) Theorell, H., *Biochem. Z.*, **279**, 463 (1935)
(*20*) Paul, K.G., *Acta. Chem. Scand.*, 5, 389 (1951)
(*21*) Willstäter, R., and Kraut, H., *Ber.*, **56**, 1117 (1923)
(*22*) Smith, L., in " Methods of Biochemical Analysis ", Interscience Publishers, Inc., New York. Vol. **II**, p. 427
(*23*) Sandell, E.B., in "Colorimetric Determination of Trace of Metal ", Interscience Publishers, New York. p. 295, p. 362 (1950)
(*24*) Green, A.A., and Hughes, W.L., in " Methods in Enzymology ", Academic Press Inc. Publishers, New York, Vol. **I**, p. 67 (1955)
(*25*) Ball, E.G., Strittmatter, C.F., and Coopper, O., *J. Biol. Chem.*, **193**, 635 (1951)
(*26*) Green, D.E., *J. Jap. Biochem. Soc.*, **29**, 65 (1957)
(*27*) Gubber, C.J., Cartwright, G.E., and Wintrobe, M.M., *J. Biol. Chem.*, **224**, 533 (1957)
(*28*) Cohen, E., and Elvehjem, C.A., *J. Biol. Chem.*, **107**, 97 (1934)
(*29*) Schultze, M.O., *J. Biol. Chem.*, **129**, 729 (1939); **138**, 219 (1941)
(*30*) Gallagher, C.H., Judah, J.H., and Ress, K.R., *Proc. Roy. Soc.Lond.*, B **45**, 134 (1956)
(*31*) Yoshikawa, H., *J. Biochem.*, **25**, 627 (1937)

The Journal of Biochemistry, Vol. 45, No. 11, 1958

CHROMATOGRAPHIC SEPARATION OF LOWER FATTY ACIDS

By TOKUICHIRO SEKI

(*From the Department of Genetics, Osaka University Medical School, Osaka*)

(Received for publication, January 24, 1958)

In the course of an investigation of the adsorption chromatography of aromatic substances on cation exchange resins (*1*, *2*), it has been found that fatty acids were as well adsorbed reversibly on H form carboxylic acid type resin from aqueous solution containing organic solvent. Elution was effected with solvent systems composed of acetone, methylethylketone and water at a ratio of 2 : 1 : 9 and 3 : 1 : 4 by volume, the former solvent being applicable to the complete separation of the lower fatty acids ranging from formic to caproic acid, and the latter to the separation of acids from acetic to lauric acid differing in chain length by two carbon atoms.

Since the acids with shorter chain-length were eluted more rapidly, the elution sequence was the same as that of reversed phase partition chromatography (*3–6*), the resin phase acting as non-polar adsorbent in this case (*7*, *8*). Although the resolving power of this method is inferior to that of gas-liquid partition chromatography (*9*, *10*), it is almost at the same level as that of liquid phase partition chromatography (*11–13*). Furthermore, since the results are quite reproducible and the procedure is simple, it is therefore suggested that this method would be particularly suited to the routine work.

EXPERIMENTAL

Chromatographic Tube—The details of chromatographic tube used in this experiment are shown in Fig. 1. Upper end of the tube is fitted with ground joint. Lower end is pulled to make a fine tip in order to pack with short fibres of glass wool. The lower end of the solvent container is also fitted with a ground joint. A plug of defatted cotton is placed in the middle of the lower stem of it, in order to remove fine particles contained in the solvent. Small amount of H form Amberlite IRC 50 resin is placed on the cotton plug to remove heavy metals contaminating the solvents.

Absorbent—Amberlite IRC 50 (analytical grade) as purchased is about 40 mesh and must be pulverized. The resin is washed with *N* sodium hydroxide on a glass filter until the filtrate becomes alkaline. The resulting sodium salt of the resin is washed with water and powdered in a ball mill without drying. The powdered resin is taken to water and passed through 200 mesh screen with running water, then filtered through 300 mesh screen with running water, then filtered through 300 mesh screen in order to remove fine particles. The screened resin is transferred on a glass filter, washed

with *N* hydrochloric acid until the filtrate is acid, and then washed with ten volumes of acetone, followed by distilled water, *N* sodium hydroxide, water and *N* hydrochloric acid according to the method of C.H.W. Hirs *et al.* (*14*).

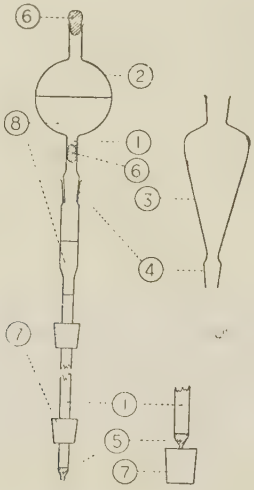


Fig. 1. Chromatographic tube.

1: Resin
2: Solvent container
3: Funnel used for the packing of the column
4: Ground glass joint
5: Glass wool
6: Cotton
7: Rubber stopper
8: Solvent

Preparation of the Column—The washed resin is equilibrated on a glass filter with the solvent described above for the usage in the chromatography and suspended by swirling in about two volumes of the same solvent (*15*). The suspension is poured into chromatographic tube through a small funnel fitted with the ground joint and allowed to settle down. When the resin has settled, the funnel is exchanged for the solvent container with the same solvent used for the packing of the column. It is specially required to maintain a few cm. of solvent layer above the surface of the column. After about 50 ml. of the solvent has been passed through, the column is ready for use.

Chromatographic Separation and Quantitative Analysis of Synthetic Mixtures—Fatty acids were dissolved in the same solvent as that used for the packing of the column and 1 ml. of the resulting solution was placed on the column. After the solution drained into the column, the inner wall of the chromatographic tube was washed with 0.1 to 0.2 ml. of the same solvent and elution was started with the same solvent. The effluent was collected by using a drop count type automatic fraction collector in fractions of 20 and/or 40 drops. For the detection of the fraction with fatty acid and quantitative analysis of the eluted acid, 0.3 ml. of phenol red pH indicator solution was added to the effluent and titrated with 0.004 *N* sodium hydroxide (*16*). Blank titration value was between 0.07 and 0.13 ml., so that the presence of 5 μM or more of fatty acid was required for the accurate determination. When the column was used several times or sodium ion was adsorbed at the top of the column, the column was washed with

the solvent containing 0.3 *N* hydrochloric acid instead of distilled water. Then it was washed with the original solvent used for the packing of the column until the effluent became neutral against thymol blue. Amberlite IRC 50 column can be used repeatedly. When the column is not used promptly after preparation, it may be preserved in the situation that the solvent container attached on the top of the tube is filled with the solvent and the flow of the solvent is stopped by pressing lightly the bottom of the tube on a rubber stopper.

RESULTS AND DISCUSSION

Typical chromatograms of fatty acids are presented in Fig. 2 and Fig 3. Relative positions of eluted acids were determined from the chromatogram

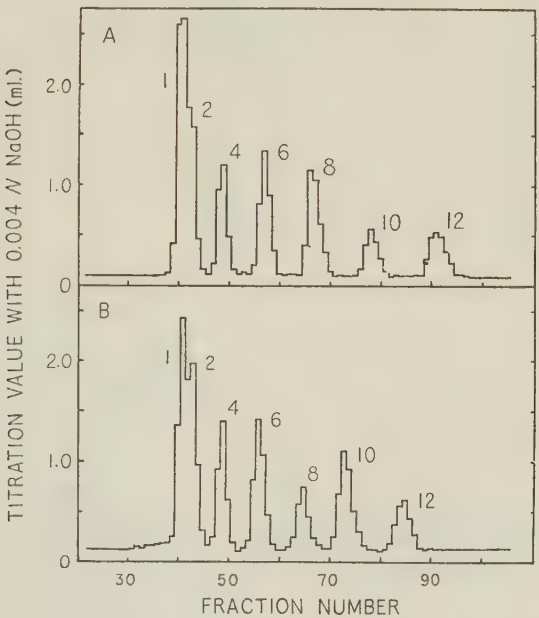


FIG. 2. Elution diagram of lower fatty acids.

One fraction: 40 drops
Flow rate: 2 drops/minute
Column size: 0.9×112 cm.
A: Added as free acid form
B: Added as sodium salt

1: Formic acid
2: Acetic acid
4: *n*-Butyric acid
6: Caproic acid
8: Caprylic acid
10: Capric acid
12: Lauric acid

of single known acid or the mixture of several known acids (*16*). When a solution containing several acids was used, each was mixed at a different ratio in order to facilitate the identification of the eluted peak. On account of the high pK_a values of fatty acids, which range from 4.75 to 4.85 with the exception of formic acid (pK_a 3.75) (*17*), dissociation of acid is surpressed

to a considerable extent in the resin phase suggesting that the fatty acid is adsorbed in the undissociated form. The recovery of the acids reported in Table I, was satisfactory, with the exception of formic acid. The lower recovery of this acid may be in part due to its low pK_a value, because with

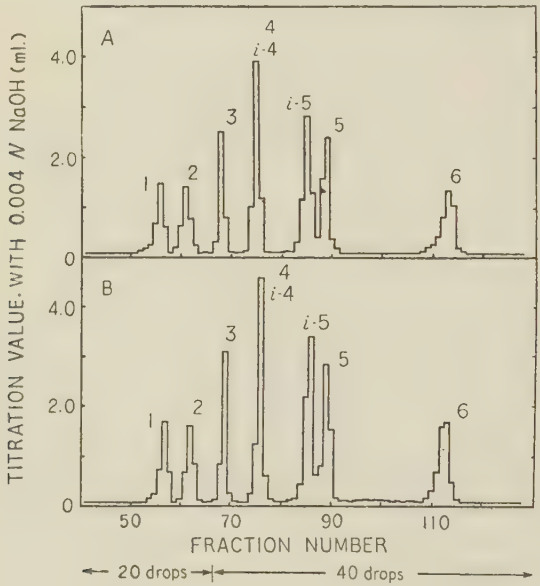


Fig. 3. Elution diagram of lower fatty acids.

Flow rate: 2.5 drops/minute
Column size: 0.76×114 cm.
A: Added as free acid from
B: Added as sodium salt
*α-Methylbutyric acid overlaped with *iso*-valeric acid.

1: Formic acid
2: Acetic acid
3: Propionic acid
i-4: *iso*-Butyric acid
4: *n*-Butyric acid
**i*-5: *iso*-Valeric acid
5: *n*-Valeric acid
6: Caproic acid

hydrochloric acid, a much stronger acid, the observed recovery was much lower. The separation was performed at 20°, although the efficiency of the column was substantially unaltered when operated at 30° except for the slight decrease in the elution volumes of the eluted acids. The capacity of the column was high and about 100 μM each of the acid could be added as the free acid form. Since the alkali salt of fatty acid is decomposed and fatty acid is set free by carboxylic acid type cation exchanger, it is noteworthy that their sodium salt dissolved in the solvent (total 100 to 300 μM) could be added to the column. Sodium ion was adsorbed at the top of the column forming a narrow zone, which could be clearly revealed as a red zone by running though the column the same solvent containing phenol red as an indicator.

The addition of the sodium salt of the fatty acid will enable the separation

of the fatty acid in the steam distillate without transfer to an organic phase.

TABLE I

Recovery of Fatty Acids from the Chromatographic Column

Composition of the solvent used	MEK[1]-Me_2CO[2]-H_2O 1:3:4		MEK-Me_2CO-H_2O 1:2:9	
Form of fatty acid added	A[3] (%)	B[4] (%)	A (%)	B (%)
Formic acid			65	70
Acetic acid	95	93	98	95
Propionic acid			90	82
iso-Butyric acid			85	90
n-Butyric acid	98	94	94	91
iso-Valeric acid			88	102
n-Valeric acid			89	90
Caproic acid	92	103	98	100
Caprylic acid	95	105		
Capric acid	100	100		
Lauric acid	85	80		

1) MEK: Methylethylketone.
2) Me_2CO: Acetone.
3) A: Added as free acid form.
4) B: Added as sodium salt.

SUMMARY

A column chromatographic method for fatty acid fractionation was described in which H form Amberlite IRC 50 was used as adsorbent and the mixture of acetone, methylethylketone and water as solvent system. It was shown that this procedure successfully separated fatty acids from formic to lauric acid differing in chain length by one or two carbon atoms. The recovery of the acids eluted ranged from 65 to 105 per cent. The elution pattern of the acids was reproducible.

The author expresses his thanks to Prof. T. Kaneko for the kind gift of *n*-valeric acid and to Nihon Yushi Co., Ltd. for the kind supply of capric and lauric acid.

REFERENCES

(*1*) Seki, T., *J. Chem. Soc. Jap.*, **75**, 1297 (1954)
(*2*) Seki, T., and Morimoto, S., *J. Chem. Soc. Jap.*, **77**, 1124 (1956)
(*3*) Howard, G.A., and Martin, A.J.P., *Biochem. J.*, **46**, 532 (1950)
(*4*) Wittenberg, J.B., *Biochem. J.*, **65**, 42 (1957)
(*5*) van de Kamer, J.H., Pikkar, N.A., Bolssens-Frankena, A., Couvée-Ploeg, C.,

and VAN Ginkel, L., *Biochem. J.*, **61**, 180 (1955)
(*6*) Vandenheuvel, F.A., and Vatcher, D.R., *Anal. Chem.*, **28**, 838 (1956)
(*7*) Green, T., Howitt, F.O., and Preston, R., *Chem. and Ind.*, 591 (1955)
(*8*) Reichenberg, D., *Chem. and Ind.*, 958 (1956)
(*9*) James, A.T., and Martin, A.J.P., *Biochem. J.*, **50**, 679 (1952)
(*10*) James, A.T., and Martin, A.J.P., *Biochem. J.*, **63**, 144 (1956)
(*11*) Cornforth, J.W., Hunter, G.D., and Popjak, G., *Biochem. J.*, **54**, 597 (1953)
(*12*) Zbinovsky, V., *Anal. Chem.*, **27**, 764 (1955)
(*13*) Corcoran, G.B., *Anal. Chem.*, **28**, 168 (1956)
(*14*) Hirs, C.H.W., Stein, W.H., and Moore, S., *J. Biol. Chem.*, **200**, 496 (1953)
(*15*) Stein, W.H., and Moore, S., *J. Biol. Chem.*, **192**, 663 (1951)
(*16*) Kinnory, D.S., Takeda, Y., and Greenberg, D.M., *J. Biol. Chem.*, **212**, 379 (1955)
(*17*) Baker, H.A., Methods in Enzymology, edited by Colowick, S.P., and Kaplan, H.O., Academic Press INC, Publishers New York, Vol. III, p. 374 (1957)

The Journal of Biochemistry, Vol. 45, No. 11, 1958

THE MECHANISMS OF CATECHINS METABOLISM

I. ACIDIC SUBSTANCES IN THE URINE OF RABBITS ADMINISTERED (+)-CATECHIN

By YASUYOSHI OSHIMA, HIROYASU WATANABE and SAKIKO ISAKARI

(*From the Laboratory of Biochemistry, Faculty of Agriculture, University of Kyushu, Fukuoka*)

(Received for publication, March 7, 1958)

Catechins occur widely in plants. Amoung our foods green tea and caccao especially contain (−)-epicatechin in large quantities. The metabolism of flavanol compounds is, however, known very little. Recently Booth *et al.* (*1*), Lang *et al.* (*2*) and Clark *et al.* (*3*) reported metabolic mechanisms of rutin and quercetin, configurations of which are closely related to catechin, though their studies were not fully accomplished.

The authors made an attempt to elucidate the metabolic mechanisms of catechin, in detail. Following experiments with (+)-catechin which is easily obtainable in pure state than any other types of catechin.

Though several kinds of aromatic hydroxy compounds might be found in the urine of mammalian animals, some new aromatic hydroxy compounds would be expected to be excreted in urine, as in case of quercetine (*1*), since catechin has two hydroxyaromatic rings in it.

In the present experiment, after feeding catechin to rabbits, hydroxyaromatic substances excreted in urine were identified as protocatechuic vanillic and *m*-hydroxybenzoic acids by paper chromatography. Using a column chromatography with cellulose powder, these substance could be isolated in crystalline form.

EXPERIMENTS AND RESULTS

Collection of Urine—Rabbits (body weight 2-3 kg.) have been kept in the carges designed to be able to separate urine and excrements. They received 0.6 g. of catechin a day per kilogram of body weight with okara (hot water-extracted residues of soya bean powder). The urine was collected for seven days in receiving flasks containing 50 ml. of 10 per cent hydrochloric acid in order to prevent oxidation of phenolic substances. Totally 2.4 liters of urine were collected.

Extraction of Acids Substance—The urine was saturated with sodium chloride, and extracted with an equal volume of ether for three times. The ether layer was separated, dried with sodium sulfate and evaporated to about 200 ml.

The saturated sodium bicarbonate solution was added into which acidic substances were transfered. The alkaline layer, after washed with ether, was acidified with hydrochloric acid, saturated with sodium chloride and extracted with 100 ml. of ether for three times. Ether was dried with sodium sulfate and evaporated. 0.8 g. of oilly residues were obtained. Control experiments were carried in the same procedure as described above, using the urine of catechin-untreated rabbits.

Paper Chromatogrphy—The ether extracts prepared as above tested by paper partition chromatography. Development was carried out with a mixture of

TABLE I

Color Reaction and R_f Values of the Substances from the Urine of Rabbits Administered (+)-*Catechin.*

Spots	Color reaction	R_f values
A	Orange-red	0.81
B	Yellow	0.41
C	Yellow	0.25
D	Red-purple	0.11
E	Red then black	0.02

TABLE II

Color Reactions and R_f Values of the Synthesized Phenolic Compounds.

Phenolic compounds	Color reactions	R_f values
3,4-Dihydroxyphenyl acetic acid	Red then black	0.04
Homovanillic acid	Red	0.62
4-Hydroxyphenyl acetic acid	Red	0.29
Protocatechuic acid	Red then black	0.03
Vanillic acid	Orange-red	0.84
4-Hydroxybenzoic acid	Yellow	0.28
3-Hydroxybenzoic acid	Yellow	0.28
Phloroglucinic acid	Yellow-brown	0.02

water saturated benzene and acetic acid (9 : 1 by volume). The paper was sprayed with a solution of freshly prepared diazotized sulfanilic acid and then followed by 20 per cent. sodium carbonate to detect the location of the vorious phenolic substances as described by Bray *et al.* (*4*).

3,4.Dihydroxyphenylacetic, homovanillic, 4-hydroxyphenylacetic, protocate-

chuic, vanillic, 3-hydroxybenzoic, 4-hydroxybenzoic and phloroglucinic acids, which were considered as possible metabolites of catechin or have been found appreciablly in mammalian urine were synthesized. The R_f values and colour

Table III

Identification of the Spot A as Vanillic Acid

Solvents	R_f values	
	Spot A	Vanillic acid
Bz. AcOH. H_2O[1)]	0.82	0.83
Xyl. AcOH. H_2O[2)]	0.58	0.58
$CHCl_3$. AcOH.H_2O[3)]	0.97	0.97

1) Above described.
2) A mixture of water saturated xylene and acetic acid (9 : 1 by volume).
3) A lower phase of a mixture of chloroform, acetic acid and water (2 : 1 : 1 by volume).

Table IV

Identification of the Spot C as Mono-Hydroxybenzoic Acid

Solvents	R_f values	
	Spot A	*m*- or *p*-Hydroxybenzoic acid
Bz. AcOH. H_2O[1)]	0.28	0.28
10 per cent AcOH	0.69	0.70
$CHCl_3$. AcOH. H_2O[3)]	0.79	0.79

1) 3) see Table III.

Table V

Identification of the Spot E as Protocatechuic Acid

Solvents	R_f values	
	Spot E	Protocatechuic acid
Bz. AcOH. H_2O[1)]	0.02	0.02
Xyl. AcOH. H_2O[2)]	0.17	0.18

1) 2) see Table III.

reactions of these compounds were examined chromatographycally by the some method.

Judging from the R_f values and colour reactions, the spots of A, C and E derived from the urine extracts were considered as vanillic, 4-hydroxybenzoic or 3-hydroxybenzoic and protocatechuic acids. These acids were further studied chromatographycally on a same paper with different systems, however, identification with 4- and 3-hydroxybenzoic acids was not feasible as shown in Table IV.

Column Chromatography—The isolation of these compounds in crystaline state was performed, using a column in which cellulose powder was uniformly packed in a glass tube of 2.2×60 cm. 0.8 g. of the acidic extracts were placed on the top of the column and elution was done with a lower phase of a mixture of chloroform, acetic acid and water (2 : 1 : 1 by volume). From the each fraction of eluted solution (each fration : 8 ml.), one drop was taken and color reaction was tested. Vanillic and mono-hydroxybenzoic acids were found in the fractions from No. 8–11 and 14–19 respectively. With this solvent system protocatechuic acid was hardly eluted out from the column. Thus ether was used to elute out this acid, and it came down in No. 31–35. Each fraction was placed in the air to evaporate chloroform and ether. The fraction tubes were then preserved in a desiccator containing sodium hydroxide in order to evaporate remained acetic acid. Crystalline substances separated in the tubes of No. 8–10, 15–17, and 32–35 were recrystallized from small amount of water respectivily. Estimated melting points of each compound were 209–210°, 200° 200° (decom.), coinciding with those of vanillic, 3-hydroxybenzoic, and protocatechuic acids. Comparing with the R_f values of chromatography and the results of mixed melting point measurement, the spots of A, C and E were thus identified as vanillic, 3-hydroxybenzoic and protocatechuic acids. Yields of these acidic substances were 8, 50 and 120 mg. respectively.

DISCUSSION

It was observed in the metabolic experiment that the side benzene ring of catechin is relatively stable, and excreated in a from of simple acid as protocatechuic acid. However, it is possible that the further degration would proceed since compared with the amount of administered catechin to rabbits, the acidic substance were excreted only in small quantities. On the other hand, derivatives of pyrone or phloroglucin ring could not be found, indicating that these rings would be relatively unstable, as compared with the side benzene ring.

Vanillic acid seems to be formed by methylation of protocatechuic acid or from previously methylated catechin. Since none of these two acids was found in control, these are evidentally considered as metabolites of catechin.

3-Hydroxybenzoic acid had been found in the urine of rabbits (5), and in our experiment it also appeared without administrating catechin. But its spot on paper chromatogram because larger on administration of catechin. So its increase is considered due to catechin. The mechanism of 3-hydroxybenzoic acid formation, whether it is secondarily produced from protocatechuic

acidor derived from other mechanisms, is a problem for further study. According to DeEds *et al.* (*6*) who reported about methylation and dehydration of phenolic compounds by rats and rabbits, oral administration of protocatechuic acid resulted in the excretion of vanillic acid, but not 3-hydroxybenzoic acid.

SUMMARY

1. In the urine of rabbits administered with (+)-catechin, five acidic metabolites (A, B, C, D and E) were recognized by paper chromatography. A, C and E were excreted in large quantities, but B and E in trace.

2. The spots of A, C and E were chromatographycally were vanillic, 3-hydroxybenzoic (or hydroxybenzoic) and protocatechuic acids respectively, and these isolated in crystalline forms using a cellulose powder column chromatography were identified by mixed melting point measurement.

REFERENCES

(*1*) Booth, A.N., Murray, C.W., John, F.T., and DeEds, F. *J. Biol. Chem.*, **223**, 251 (1956)
(*2*) Lang, K., and Weyland, *Biochem. Z.*, **327**, 109 (1956)
(*3*) Clark, W.G., and Mackay, E.M., *J. Am. Med. Asoc*, **143**, 1411 (1950)
(*4*) Bray, H.G., Thorpe, W.V., and While, K., *Biochem. J.*, **46**, 271 (1950)
(*5*) Boyland, E., Maura, D., Solomon, J.B., and Wiltshire, G.H., *Biochem. J.*, **53**, 420 (1953)
(*6*) DeEds, F., Booth, A.N., and Hones, F.T., *J. Biol. Chem.*, **225**, 615 (1957)

The Journal of Biochemistry, Vol. 45, No. 11, 1958

STUDIES ON HEMOGLOBIN

II. ARGININE-CONTAINING PEPTIDES FROM THE HYDROLYSATE BY STREPTOMYCES GRISEUS PROTEINASE

By SHIGERU SASAKAWA* and KAZUO SATAKE

(From the Department of Chemistry, Faculty of Science, Tokyo Metropolitan University, Tokyo)

(Received for publication, April 28, 1958)

It has been well known for a number of years, that hemoglobin may be converted to subunits approximating half-molecules under the action of urea (*1*), dilute acid (*2*), surface denaturation (*3*) or strong salt solutions (*4*). Further evidences for the existence of hemoglobin as a dimer are derived from the X-ray studies of Perutz and his colleagues (*5*), who have shown diad axes of symmetry, and from the sulfhydryl group analysis by Ingram (*6*), who has shown that these groups in various hemoglobins occour in multiples of two. According to Gutter and Peterson (*7*), however, hemoglobin is dissociated into four subunits by the action of concentrated urea solution containing mercaptoethanol, which suggests that hemoglobin is a tetrad of myoglobin-like substances, containing only one hemin as the prosthetic group (*8*).

In the previous paper on the N-terminal amino acid sequences of a variety of hemoglobins, one of the authors (Satake) reported that the protein was composed of two or three different pairs of peptide chains, assuming the molecular weight to be 68,000. Thus horse hemoglobin has three pairs of chains, (A : Val-Leu-)$_2$ (B : Val-Gly-)$_2$ (C : Val-Glu-**)$_2$*** and bovine hemoglobin has two pairs, (A : Val-Leu-)$_2$ (B : Met-Gly-)$_2$ (*9*). These findings suggest that the protein A_2B_2 (or $A_2B_2C_2$) dissociates into two pieces of AB (or ABC) then sometimes into four pieces, of two A and two B, although the presence of three different N-terminal chains A, B and C in the horse hemoglobin molecule complicates the considerations for the dissociation of this protein into four subunits (*8*, *10*).

However, there has been no direct ercidence as to whether hemoglobin

---

* Maiden name: SHIGERU NAKADATE.

** The abbreviations of Brand (*11*) are used to present the name of amino acid residues.

*** Hemoglobin from most animals has Val-Asp- instead of Val-Glu-, and Val-Gly- instead of Met-Gly-. In general, the amino acid sequence of a protein from different source seems to have a foundamental form except some amino acid residues, which are replaced by the other abnormal ones due to the species and family differences (*8*, *12*).

has a pair of similar amino acid sequences in the molecule or not, except in the case of the N-terminal one. Therefore it is perdinent to note that some arginine peptides could be isolated in the yield of 2 mole per mole ($68{,}000 \times g$) from the hydrolysates of horse and bovine hemoglobins by *Streptomyces griseus* proteinase, which was recently purified from the mould culture fluid in the crystalline state by Nomoto (*13*).

EXPERIMENTALS

Materials—Horse and bovine globins were obtained from the corresponding hemoglobins, which were prepared according to the same method as described in the previous paper (*10*) by the treatment with hydrochloric acid-containing acetone (*14*). The air-dried preparation contained about 12 per cent of moisture, (Kjeldahl-N 15.9–16.1 per cent), thus 75 mg. corresponded to 1 μM of hemoglobin. The purified preparation of *Streptomyces griseus* proteinase (*13*) was kindly sent by Dr Nomoto.

Methods: *Hydrolysis*—Two mg. of the proteinase was dissolved into the solution of 75 mg. globin in 15 ml. of *M*/50 phosphate buffer (pH 7.5) and the solution was kept at 25° for 10 hours. According to the extinction values of amino group after N-picrylation by picrylsufonate (*15*), there was only a slight increament of amino-N after three hours under these conditions.

Chromatographic Separation of Arginine Peptides—The hydrolysate, after concentrating to 1 ml, was added to a column of Amberlite IRC-50 (1 cm.^{2} × 60 cm. 150–250 mesh), previously equilibrated with borate buffer (pH 8.1) consisting of 3 parts of *M*/20 borax and 7 parts of *M*/5 boric acid. The column was eluted with sodium chloride in the same buffer at a rate of 5 ml. per hour while increasing the concentration of sodium ion from 0.3 to 0.5 and then to 0.7 g. ion per liter (*16*). The effluent was collected in 3.0±0.05 ml. portion by an automatic fraction collecter.

Spectro Photometric Determination of Arginine Peptides—The concentration of arginie peptides was determined by the modified Sakaguchi reaction using 8-hydroxyquinoline (*17*), assuming that the arginine peptide gave the same color intensity as the equivalent free arginine. (See Table I).

Purification of Arginie Peptides—The fractions corresponding to each peak on the elution diagram, previously described (Fig. 1) were passed through a column of Amberlite IRA-410 (OH-from; 1 × 10 cm.) at a rate of 0.5 ml. per minute to remove the contaminated nonarginine peptides on the column. The combined filtrate and washings (50 ml.), after concentration to a few ml., was treated with 1 ml. of 1 per cent fluoro-2, 4-dinitrobenzene in methanol and 1 ml. of 1 per cent sodium bicarbonate for 5 hours at room temperature to dinitrophenylate the arginine peptides (*18*, *19*). The acidified mixture (pH < 1) after washing three times with peroxide free ether; 3 ml. in each portion to remove the ether-soluble DNP-derivatives was passed through a column of talc. celite (1 : 1) (1 × 10 cm.) in order adsorb DNP*-arginine peptide present in the aqueous solution. The adsorbed yellow band was well washed with *N* HCl (50 ml.) to remove inorganic salt; then was eluted with an equal volume mixture of ethanol and *N* HCl (*18*).

Determination of the Structure of Arginine Peptides—An aliquot of the eluate from talc-column, was hyrolysed with 6 *N* HCl at 110° for 5 hours, and the produced DNP-amino acids and free amino acids were identified by paper chromatography; *n*-butanol-

* DNP: 2, 4-dinitrophenyl.

saturated with 0.1 per cent ammonia and 1.5 *M* phosphate buffer (pH 6) (*18*) were used as the solvent for DNP-amino acids; phenol saturated with water and *n*-butanol-acetic acid-water (4 : 4 : 2 *v/v*) were the solvent system for free amino acids (ninhydrin positive).

Another aliquot of the eluate was also used for the determination of the ratio of Sakaguchi reaction color density against the optical density at 355 mμ (in 1 per cent HCl) due to the DNP-group.

Table I

Color Intensity of the Sakaguchi-Reaction with Arginine and Its Derivatives

Fraction No[1)]	Color intensity a	b
16 (A)	.612	.596
39 (B)	.288	.302
60 (C)	.240	.214
104 (D)	.552	.538

1) The No. corresponds th the Fraction No. in Fig. 1.

a) 0.5 ml. of sample solution, after adding 0.5 ml. of 2 *N* NaOH and 0.5 ml. of 0.01 per cent 8-hydroxyquinoline was oxidized with 0.5 ml. of alkaline sodium hydrobromide (one gram of bromine was dissolved in 100 ml. of *N* NaOH) a few minutes after the addition of alkaline hydroxyquinoline. The produced red color was stabilized by the addition of 40 per cent urea solution, 30 seconds after the addition of the oxidizing agent. The solution was assayed at 490 mμ after 10~15 minutes.

b) 1.0 ml. of sample solution, after being heated at 110° for 10 hours with 10 ml. of 12 *N* HCl in a sealed tube, was dried in the desiccator containing solid sodium hydroxide. The residue was then dissolved in 1.0 ml. of distilled water. 0.5 ml. of the solution was assay by the same procedures as shown in (a).

RESULTS

As shown in Fig. 1, from the *Streptomyces griseus* proteinase digest of horse hemoglobin four kinds of Sakaguchi reaction positive components, A, B, C and D were clearly separated on a column of Amberlite IRC-50 (pH (8.1). Judging from its position on the elution diagram, A seems to be an arginine peptide containing acidic amino acids, B and C containing neutral, non-aromatic amino acid and D corresponding to free arginine. The amounts of A, B and C were about 2 μM and that of D about 8 μM per 1 μM (68×10^3 μg.) respectively and the sum was 95 per cent of the Sakaguchi reaction positive substances

* The proteinase itself contained some arginine residues, although the color density due to these was somewhat less about one third than that due to the corresponding amounts of free arginine.

in the original hydrolysate* (Table II).

The fractions corresponing to each peak gave several minor ninhydrin positive-Sakaguchi-reaction negative spots besides a major spot which ninhydrin positive-Sakaguchi positive (A~B), whose R_f values are shown in Table II. The former contamiants were easily removed by passage through a column of anionic resine, Amberlite IRA-410, then by fractionation of the DNP-derivatives with ether and with talc column, and 15~30 per cent of the main components were lost during these procedures.

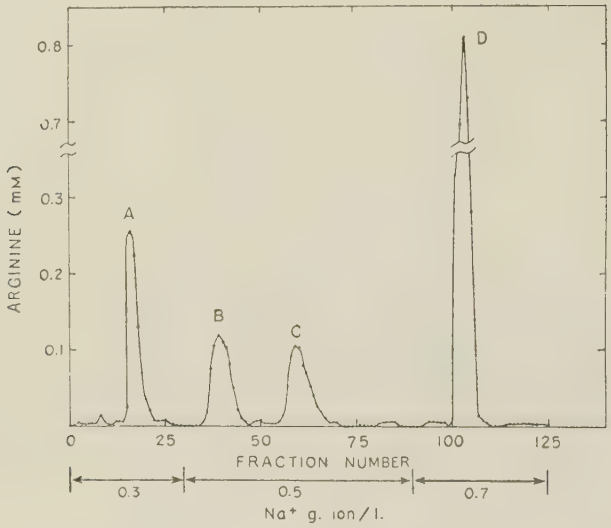


Fig. 1. Elution diagram of Sakaguchi-reaction positive components in the hydrolysate.

Sample: The hydrolysate corresponds to 75 mg. of horse globin. Colume: Amberlite IRC-50 (pH 8.1) 1 cm.^{2}×60 cm. Solvent: NaCl in borate buffer (pH 8.1).

These chromatographically pure DNP-components gave a ratio of the Sakaguchi-reaction color against the optical density at 355 mμ due to DNP-group, similar to DNP-arginine within the range of five per cent, indicating these components were free from non-arginine peptide, and had one arginine residue in the molecule. Their structure may be elucidated as follows;

A: Arg-Glu, B: Ala-Arg,
C: Gly-Arg, D: Arg

from the results of the analysis of their amino acid constituents, which are summarized in Table II.

On the hydrolysis of bovine globin by the same proteinase, the same results were obtained. The amounts of the four components estimated from the elution diagrams, are shown in Table II together with the results with horse globin.

Table II

The Yield, Properties and Constituents of Each Fraction

Fr. No.	Yield[1] Horse Hb	Yield[1] Bovine Hb	R_f[2]	Color[3]	Sakaguchi[4]/ DNP	Amino acid constituents[5]
A	1.96	2.08	0.21	Nurple weak	0.95	DNP-Arg, Gl
B	1.95	1.93	0.09	Purple	0.96	DNP-Ala, Ar
C	2.01	2.1_2	0.03	Yellow then purple	0.98	DNP-Gly, Ar Dinitropheno
D	7.83	7.9_6	0.11	Red purple	1.02	DNP-Arg
Total	13.7_5	14.0_9				

1) The figures, expressed in mμ, show the yield from the hydrolysate corresponding to 1 μmol of hemoglobin (calculated from the elution diagrams as Fig. 1).

2) Developed with *n*-butanol-acetic acid- water (4 : 1 : 2 *v/v*)

3) Color with ninhydrine on filter paper.

4) The relative ratio of Sakaguchi-reaction color against the optical density at 355 mμ (in 1 per cent HCl) of each fraction, taking that of DNP-Arg as 1.00.

4, 5) Measured as DNP- derivatives, at the stage of talc-column effluent.

The absence of tryptophan residues was verified by Hopkins-Cole reaction.

Table III

Substrate Specificity of Streptomyces griseus Proteinase

Substrate		Substrate	
Asp-Arg (a)	+	Gly-Gly (b)	−
Glu-Arg (a)	+	Gly-Ser (b)	−
Gly-Arg (a, b)	−	Gly-Val (b)	−
Ala-Arg (a, b)	−	Gly-Asp (b)	−
Val-Arg (a)	+	Ala-AspNH_2 (b)	−
Leu-Arg (a)	+	Leu-Tyr (b)	+
His-Arg (a)	+	Gly-Pro (b)	−
Arg-Glu (a, b)	−		

The symbol + (−) indicates that the peptide bond is able (unable) to be hydrolysed.

a) From the experiments using globin as the substrate.

b) 1 ml. of 10^{-3} *M* peptide solution in *M*/50 phosphate buffer (pH 7.5) was incubated with 2 μg. of the proteinase at 25° for 10 hours. And the formation of splitt products was determined by paper chromatographic analysis. The peptide designated as (+), was completely hydrolysed under these conditions, whereas those designated as (−) gave no trace of amino acids.

DISCUSSION

From the above results, it is concluded that horse and bovine hemoglobins have 14 arginine residues, and six of them at least, are present as a pair of Arg-Glu, Ala-Arg and Gly-Arg. If there occured no new synthesis of peptide bonds due to transpeptidation, this seems to be a valid assumption under in this experiment. The presence of these sequences in more than 2 moles per mole shall be excluded, as the *Streptomyces* proteinase has strong peptidase activity as well as proteinase activity and three dipeptides were verified not to be hydrolysed under these conditions.

According to Nomoto (*13*), the proteinase hydrolyses such a wide range of peptide bonds, that more than 70 per cent of those in hemoglobin can be split, althogh the precise substrate specificity has remained unknown. Recently one of the authors (Satake) (*10*) was able to establish the presence of Asp-Arg, Glu-Arg, Val-Arg, Leu-Arg and His-Arg sequences in bovine globin, besides Gly-Arg, and Ala-Arg, therefore, to some extends, its specificity is indicated in Table III, which also contains the results of its action on some peptides.

The presence of three pairs of sequence of arginine residues is not inconsistent with the molecular model composed of two similar subunits, which have been proposed for hemoglobin, although far being proven.

SUMMARY

Horse and bovine hemoglobins were concluded to have 14 arginine residues, six of which are present as pair of Arg-Glu, Ala-Arg and Gly-Arg peptides respectively, from the quantitative analysis of arginine residues in the hydrolysate by *Streptomyces* proteinase.

The authors wish to express their gratitude to Dr. Nomoto for his gift of *Streptomyces* proteinase.

The expenses of this work were in part defrayed by a Grant in Aid for Scientific Research from the Ministry of Education.

REFERENCES

(*1*) Steinhardt, J., *J. Biol. Chem.*, **123**, 543 (1938)
(*2*) Gralén, N., *Biochem. J.*, 1907 (1939)
(*3*) Guastalla, J., *Compt. rend. trau. lab. Carlsberg, Ser. Chem.*, **208**, 1078 (1939)
(*4*) Gutfreund, H., *Hemoglobin*, edited by Roughton, F.J.W. and Kendrew, J.C., Interscience, New York p. 197 (1947)
(*5*) Perutz, M.E., Linquori, A.M., and Eirich, E., *Nature*, **167**, 929 (1951)
(*6*) Ingram, V.M., *Biochem. J.*, **59**, 653 (1955)
(*7*) Gutter, F.J., Peterson, E., 128 th Meeting, of American Chemical Socibal, Minneapolis, p. 426 (1955)
(*8*) Anfinsen, C.B., and Redfield, R.R., *Advances in Protein Chem.*, **11**, 46 (1956)
(*9*) Ozawa, H., and Satake, K., *J. Biochem.*, **42**, 641 (1955)
(*10*) Ozawa, H., Tamai, K., and Satake, K., *J. Biochem.*, in press.

(*11*) Brand, E., *Ann. New York Acad. Sci.*, **47**, 187 (1946)
(*12*) Satake, K., *J. Biochem.*, will be published. See also Kusama, K., *J. Biochem.*, **44**, 375 (1957)
(*13*) Nomoto, (9 th Meeting, Tokyo), *Symposium on Enzyme Chemistry* **I**, 24, (1956)
(*14*) Jope, E.M., *Nature*, **164**, 622, (1949)
(*15*) Satake, K., Okuyama, T., Ohashi, M., unpublished.
(*16*) Ichii, S., *Biochim., et Biophys. Acta*, in press.
(*17*) Kraut, H., Schrader-Beilstein, E.V., and Weber., *Z. Physiol. Chem.*, **286**, 248 (1950); see also Sakaguchi, S., *J. Biochem.*, **37**, 231 (1950): **38**, 91, (1951)
(*18*) Sanger, F., *Biochem. J.*, **45**, 563 (1949)
(*19*) Frankel-Conrat, H. Harris, J.I., and Levy, A.L., *Methods of Biochemical Analysis*, **2**, 359 (1956)

The Journal of Biochemistry, Vol. 45, No. 11, 1958

STABILITY OF NUCLEIC ACID SYNTHESIZED IN THE PRESENCE OF CHLORAMPHENICOL IN E. COLI B UNDER GROWING AND RESTING CONDITIONS

By TADAO HORIUDHI, SUMIKO SUNAKAWA and DEN-ICHI MIZUNO

(*From the Department of Biochemistry, National Institute of Health, Tokyo*)

(Received for publication, March 14, 1958)

It has recently been shown by several authors (*1-6*) that bacterial proteins and nucleic acids once formed were conserved and did not degrade so extensively in growing condition. On the other hand, degradation and renewal in resting bacteria were shown (*7*, *8*) in contrast with those in growing condition.

We reported briefly (9) that *E. coli* RNA synthesized in the presence of chloramphenicol (CM)* degraded in resting condition. Neidhardt and Gros (*10*) and Hahn *et al.* (*11*) also found this phenomenon independently and demonstrated that this nucleic acid was degraded even in growing condition.

The present work deals with the stability difference between this RNA in growing and in resting conditions using *E. coli* B. Evidences were obtained revealing conservation of this RNA under growing condition and degradation under resting condition.

EXPERIMENTAL

Organisms—The strains used were *Escherichia coli* B and its auxotrophic mutants, tryptophan-requiring (A-5) and uracil or cytosine-requiring (F-2) isolated by us.

Chemicals—Chloramphenicol (CM) was obtained by Sankyo Co. Ltd. and used in a concentration of 30 μg. per ml. in all experiments where used (*12*). The carrier free P^{32} was supplied by Japan Radioisotope Association (originally furnished by the Radiochemical Center, Amersham, England).

Medium—The medium used was composed of the following; 1.5. KCl, 5.0 g. NaCl, 0.25 g. $MgSO_47H_2O$, 0.01 g. $CaCl_2$, 5.0 g. sodium lactate and 5.0 g. Difco vitamin-free casamino acids per litre, brought to pH 7.0 with NaOH and sterilized at 100° three times. This medium contained 20 μg. P per ml. In the case of A-5 and F-2 100 μg. per ml. of L-tryptophan and 20 μg. per ml. of uracil, respectively, were added to the above casamino acids medium. These were used for the precultures. In the final test medium for A-5 and F-2 50 μg. per ml. of L-tryptophan and 7 μg. per ml.

* The following abbreviations will be used: chloramphenicol (CM), ribonucleic acid (RNA), desoxyribonucleic acid (DNA), optical density (OD).

of uracil were used, respectively.

Bacterial Cultures and Chloramphenicol Treatment—Bacteria in exponential growth phase were used for CM treament. To obtain an adequate exponential culture the organisms were grown overnight in the casamino acids medium at 37° with aeration by shaking and transferred to a fresh medium by an inoculum of a radio 1 : 50 to allow the organism to grow for additional 3 to 4 hours at 37° with aeration by shaking.

During the exponential growth thus obtained CM was added in the concentration of 30 μg. per ml. and incubation was continued for a time shown in the experimental part.

Washing of the Cells—The cells were poured into a tube containing pieces of ice and centrifuged down in the cold with an ultracentrifuge (15,000 r.p.m. for 1 minute; Spinco Model L), washed twice with the ice cold casamino acids medium and resuspended in an equal volume to the original culture. The supernatant fraction thus obtained contained less than 0.5 per cent of the total viable cells.

Measurement of Bacterial Growth—Bacterial growth was measured by Coleman spectrophotometer and expressed in terms of the optical density (OD) at a wave length of 550 mμ. In some cases viable counts of the cells were used. The OD of the culture immediately after the washing was high compared with that before washing, but the OD decreased to be amost constant in 2–3 minutes shaking. (probably it is due to an unspecific alteration of aggregated state of the cells). We took this constant value for the starting OD immediately after the washing.

Nuclic Acid Determination—Aliquots of bacterial culture were precipitated in the 5 per cent (final concentration) trichloracetic acid. The precipitated DNA was estimated by the diphenylamine method (*13*) using calf thymus DNA as a standard and the precipitated RNA was estimated by the orcinol reaction (*14*) using D-ribose as a standard. DNA-P^{32} and RNA-P^{32} were fractionated by Hershey's procedure (*15*) with the addition of a process of lipid elimination by ether-alcohol (originally Shchmidt and Thannhauser's method).

RESULTS

Conservation of Physiological RNA and DNA under Growing and Resting Conditions—In the early exponential growth phase of culture of A-5 strain (tryptophan-requiring) P^{32} was added and incubation was continued for additional 1 hour. Cells were harvested, washed and resuspended in the casamino acids medium containing tryptophan and no P^{32}. As shown in curve b, in Fig. 1, bacterial cells began to grow immediately without delay and the RNA-P^{32} and DNA-P^{32} were conserved almost perfectly under this growing condition. In the case of strain B, the same results were obtained as those described by Hershey (*2*).

If cells of A-5 strain were resuspended in the casamino acids medium containing no tryptophan nor P^{32} after the washing, RNA-P^{32} and DNA-P^{32} also hardly degraded under this resting condition. In this resting conditions, where complete tryptophan starvation did not occur, total RNA and DNA determined by orcinol and by diphenylamine, respectively, were increased slightly, though to a negligible extent when compared with that in growing culture. F-2 (uracil or cytosine-requiring) showed the same results in tests for the role of uracil.

Conservation of RNA and DNA Synthesized in the Presence of CM under

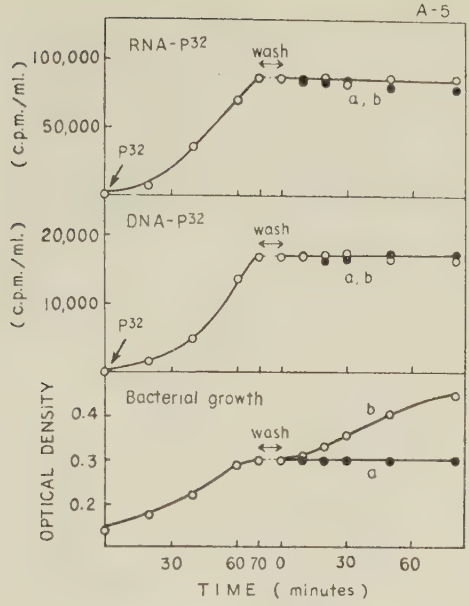


FIG. 1. Conservation after the washing of RNA-P^{32} of A-5 (tryptophanrequiring under resting and growing conditions.

Before the washing: tryptophan (+) P^{32} (+)

After the washing: *Curve a* tryptophan (−) P^{32} (−) *Curve b* tryptophan (+) P^{32} (−)

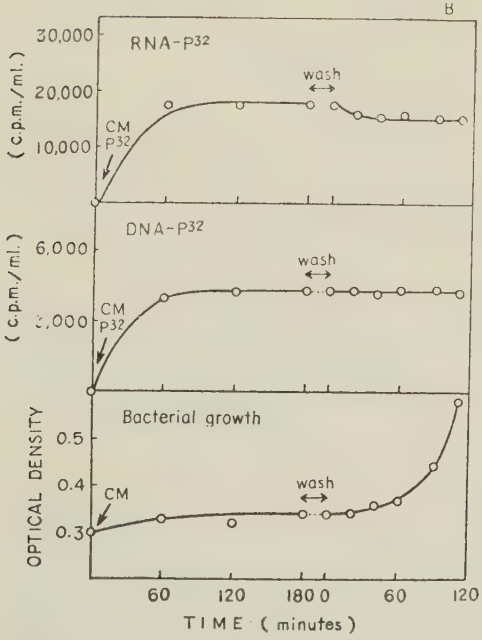


FIG. 2. Conservation after the washing of RNA-P^{32} and DNA-P^{32} of prototroph *E. coli* B pretreated with CM.

Before the washing: CM (+) P^{32} (+)

After the washing: CM (−) P^{32} (−)

Growing Condition—CM (final concentration 30 μg. per ml.) was added in the exponential growth culture of strain B followed by the addition of P^{32} after 2 minutes (*12*). The rate of P^{32} incorporation into RNA and DNA fraction

in the presence of CM was identical to that in the physiological growth within 30 minutes after the addition of CM, but in the addition 30 minutes the incorporation was not observed. Within this 1 hour after the addition of CM, the OD was increased to 1.2 fold. The total content of RNA and DNA at this stage was twice as much as those before the addition of CM. When the cells were suspended in the casamino acid medium containing on CM after the washing, a lag period was observed before an increment of OD. Fig. 2 indicates occurrence of little degradation of DNA-P^{32} as well as of RNA-P^{32} under growing condition.

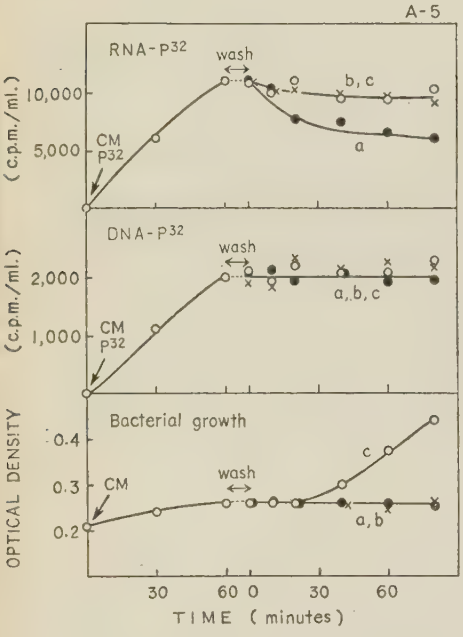


Fig. 3. Degradation and conservation after the washing of RNA-P^{32} and DNA-P^{32}, respectively, of A-5 (tryptophan-requiring) pretreated with CM.

Before the washing: CM (+) P^{32} (+) tryptophan (+)

After the washing: *Curve a*, CM (−) P^{32} (−) tryptophan (−) *Curve b*, CM (+) P^{32} (−) tryptophan (−) *Curve c*, CM (−) P^{32} tryptophan (+)

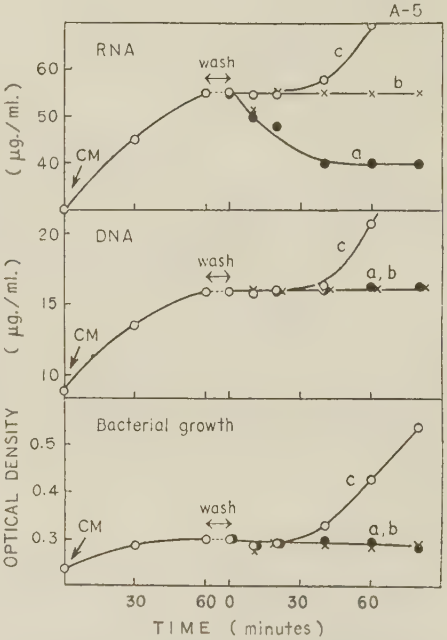


Fig. 4. Degradation and conseruation after the washing of the total RNA and DNA, respectively, of A-5 (tryptophan-requiring) pretreated with CM. Conditions are the same in Fig. 3.

Degradation of RNA and Conservation of DNA under Resting Condition after Synthesized in the Presence of CM—Experiments were carried out to ascertain whether the RNA and DNA synthesized in the presence of CM were degraded or conserved after the washing under resting condition using A-5 (tryptophan-requiring) and F-2 (uracil or cytosine-requiring). CM was added in the

logarithmic growth culture with or without P^{32} of A-5 which was kept incubating for an hour with aeration. The culture was washed to remove CM and P^{32} (in case of its presence) and resuspended in the P^{32}-free medium containing the following combination of the components: casamino acids medium (Curve a in Figs. 3 and 4), casamino acids medium plus CM (Curve b) and casamino acids plus tryptophan (Curve c).

Results are depicted in Fig. 3 which shows the stability of P^{32} incorporated into nucleic acids and in Fig. 4 which indicates that the same experiment was confirmed by orcinol and diphenylamine determination. In these experiments the resting condition will be shown in curves a and b, whereas curve c will represent the growing condition. Remarkable points in Figs. 3 and 4 are

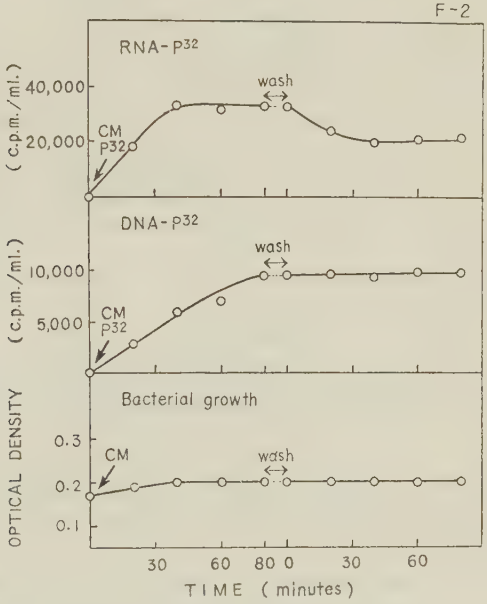


Fig. 5. Degradation and conservation after the washing of RNA-P^{32} and DNA-P^{32}, respectively, of F-2 (uracil or cytosine-requiring).

Before the washing: CM (+) P^{32} (+) uracil (+)
After the washing: CM (−) P^{32} (−) uracil (−)

that RNA synthesized in the presence of CM was degraded by 50 per cent or more in resting condition (Curve a), whereas it was conserved in the presence of CM (Curve b) or in growing condition (Curve c). In the case of growing cultre RNA and DNA increased after a lag period and P^{32} in the RNA and DNA fractions were conserved as previously shown in Fig. 2. The viable counts of the cells under the resting condition after the washing were decreased only by about 5 per cent in 90 minutes. It is hardly accepted that the degradation of the RNA synthesized in the presence of CM depended

mainly on the lysis of bacteria, since the RNA of curve b and DNA (Curves a, b and c) were conserved.

The same experiment using F-2 strain and taking the absence of uracil as a limiting factor showed the results identical to those using A-5 (Fig. 5). In this case P^{32} incorporated into RNA being degraded was released gradually into the medium concurrent with the gradual increase of OD at 260 mμ in the same fraction. It will suggest that the degradation of the RNA may occur at the nucleotide level releasing them into the medium.

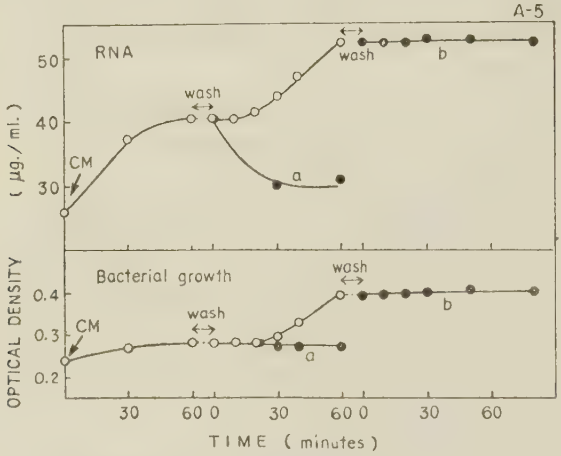


Fig. 6. Stabilization of the RNA synthesized in the presence of CM, of A-5 (tryptophan-requiring) after 1 hour exposure to tryptophan.

Before the first washing: CM (+) tryptophan (−)

After the first washing: *Curve a*, CM (−) tryptophan (−)

Between the first washing and the second washing: CM (+) tryptophan (+)

After the second washing: *Curve b*, CM (−) tryptophan (−)

Stabilization of the RNA Synthesized in the Presence of CM during Growing Stage—As indicated in the preceding sections the RNA synthesized in the presence of CM was degraded under resting condition and conserved under growing condition. Then a question arises whether the RNA which would have been degraded in the resting stage can be conserved as such to be degraded or can be converted into a different stable from during the growing stage after the washing. The RNA of A-5 synthesized in the presence of CM was tested for stability. After the washing of cell suspension in which the RNA was synthesized in the presence of CM, the cells were resuspeneded in the casamino acids medium plus tryptophan. After 60 minutes when the cells were growing they were washed and resuspended in the tryptophan deficient casamino acids medium. As shown in Fig. 6 no degradation of RNA was observed, whereas the RNA in the resting stage just after the washing showed

a marked degradation by more than 50 per cent. It will suggest that th RNA was converted into a more stable from during the growing stage.

DISCUSSION

Neidhardt and Gros (*10*) brought to light that the RNA synthesize in the presence of CM degraded in the resting stage and that, if CM wa added in this resting stage, the special RNA was resynthesized showing a increase of C^{14}-adenine in the RNA fraction. Hahn *el al.* (*11*) succeeded i demonstrating the cytological change during the whole course of the CM treatment. The present data have shown that the RNA was degraded in th resting stage but conserved in the growing stage using B strain and its aux otrophic strains in a casamino acids medium. Neidhardt and Gros ab served 20 per cent or more degradation of the RNA even under growin condition and Hahn's data showed that both the RNA and DNA wer pegraded even under growing condition using B/r strain in a mineral medium These inconsistencies may be due to the differences of the media used rathe than due to the differences of the strains used. The media they used wer mineral medium, while the casamino acids medium was used in the presen experiment. Thus the nutritional conditions of the cells were quite differen from each other. The marked decrease of the viable counts described b Hahn *et al.* after 3 hours exposure to CM (10 μg. per ml.) using *E. coli* was not observed in our medium. In the casamino acids medium used b us, viable counts of cells were increased by 20 per cent or more after CM treatment (1 hour, 30 μg. per ml.) without showing detectable increment c protein nitrogen as determined by Nesslerization and were not changed durin an additional 2 hours. Cells to B strain treated with CM for 1 hour wer smaller but thiches than normal cells in shape as visualized by electron micro scope.* The concentration of CM, 30 μg. per ml. in this nutrient medium had an activity to stop the synthesis of protein almost completely but to allo cell division when cells were in the stage of division. The short lag period i the present work after the washing of cells to eliminate CM compared t other papers can also be explained by the difference of the state of cell Therefore, it can be considered that in a nutrient medium the RNA an DNA synthesized in the presence of CM can be conserved under the growin condition, although a different nucleic acid from the normal was obtaine (Pardee *et al.* (*16*)) and the base composition thereof is identical with th normal (Lombard and Chargaff (*17*), Pardee and Prestidge (*18*)).

The fact that the RNA can be conserved under growing condition can b explained by the following alternatives: (1) degradation does occur but th fragments released synthesized normal nucleic acid immediately after the degra dation or (2) degradation does not occur. If the first hypothesis holds valid the RNA would have shown a constant value or otherwise the DNA and th protein would have been increased concurrent with the RNA degradation i

* Unpublished observation.

Fig. 5, where F-2 strain was used and the degradation fragments of nucleic acid released, of any, would have been reutilized immediately by this strain. In fact the RNA-P^{32} was degraded and the DNA-P^{32} was conserved. The same results were obtained using orcinol and diph enylamine analysis for the total nucleic acid contents. Therefore, the first alternative can be neglected and the second alternative may be true.

If the RNA is conserved as such under growing condition, it should have been degraded when the condition converts to a resting stage. As seen in Fig. 6 the RNA having experienced the growing condition for 60 minutes does not degrade even in resting stage. The mechanism for stabilization of the RNA is obscure. It may be considered that stabilization in the polymer level does occur in view of the high amount of oligonucleotides accumulated in the cells (Pardee *et al.* (*16*)) or that the stabilization through the combination with some protein to form a nucleoprotein can occur. The stabilized RNA may be diluted to normal level by followed cell divisions.

The time needed for stabilization can be elucidated in the following experiment. The cells of A-5, after the RNA synthesis in the presence of CM and the washing were suspended in the growing medium, washed again after 0, 10, 20, 30 and 60 minutes and resuspended in the tryptophan deficient medium. The degradation of RNA was observed by 74 per cent for 0 minutes, 52 per cent for 10 minutes, and 5 per cent for 20 minutes taking 100 per cent for the RNA synthesized in the presence of CM and no degradation was observed after 30 and 60 minutes where the bacterial growth was observed. Therefore, the stabilization of a major part of the RNA occurred during the lag period of the bacterial growth.

Finally, these RNA and DNA synthesized in the presence of CM and melibiose after β-galactosidase permease formation did not participate in, nor accerelate, either the β-galactosidase or total protein synthesis in our system of growing condition as previously reported (*9*).

SUMMARY

1. The stability of the RNA and DNA synthesized in the presence of CM were examined using *E. coli* B and its auxotrophic mutants: tryptophan-requiring (A-5) and uracil or cytosine-requiring (F-2). It has beed shown that these RNA and DNA were conserved under growing condition in the casamino acids medium.

2. This RNA was degraded by 50 per cent or more under resting condition where tryptophan and uracil were limiting factors using A-5 and F-2, respectively, but the conservation of this DNA was observed under the same condition.

3. This RNA was stabilized by the exposure to a growing stage during the lag time of bacterial growth, and after that this RNA showed on degradation even in case the limiting factor was removed.

4. The stability of the RNA between the growing and the resting condition was discussed.

REFERENCES

(*1*) Koch, A.L., and Levy, H.R., *J. Biol. Chem.*, **217**, 947 (1955)
(*2*) Hershey, A.D., *J. Gen. Physiol.*, **38**, 145 (1954)
(*3*) Manson, L.A., *J. Bact.*, **66**, 703 (1953)
(*4*) Labaw, L.W., Moseley, V.M., and Wyckoff, R.W.G., *J. Bact.*, **59**, 251 (1950)
(*5*) Rotman, B., and Spiegelman, S., *J. Bact.*, **68**, 419 (1954)
(*6*) Hogness, D.S., Cohn, M., and Monod, J., *Biochim. et Biophys. Acta*, **16**, 99 (1955)
(*7*) Mandelstam, J., *Nature*, **179**, 1179 (1957)
(*8*) Halvorson, H., *Bacteriol. Proc.*, 137 (1957)
(*9*) Horiuchi, T., Sunakawa, S., and Mizuno, D. The 30th General Meeting of the Japanese Biochemical Society, July 14, 1957, at Kyoto Medical University, *J. Jap. Biochem. Soc.* (*in Japanese*), **29**, 642 (1957)
(*10*) Neidhardt, F.C., and Gros, F., *Biochim. et Biophys. Acta*, **25**, 513 (1957)
(*11*) Hahn, F.E., Schaechter, M., Ceglowsky, W.S., Hopps, H.E., and Ciak, J., *Biochim. et Biophys. Acta*, **26**, 469 (1957)
(*12*) Tomizawa, J., and Sunakawa, S., *J. Gen. Physiol.*, **39**, 553 (1956)
(*13*) Dische, Z., *Microchemie*, **8**, 4 (1930)
(*14*) Kerr, S.E., and Seraidarian, K., *J. Biol. Chem.*, **159**, 211 (1945)
(*15*) Hershey, A.D., *J., Gen. Physiol.*, **37**, 1 (1954)
(*16*) Pardee, A.B., Paigen, K., and Prestidge, L.S., *Biochim. et Biophys. Acta*, **23**, 162 (1957)
(*17*) Lombard, A., and Chargaff, E., *Biochim. et Biophys. Acta*, **25**, 549 (1957)
(*18*) Pardee, A.B., and Prestidge, L.S., *J. Bacteriol.*, **71**, 677 (1956)

The Journal of Biochemistry, Vol. 45, No. 11, 1958

DENATURATION AND INACTIVATION OF ENZYME PROTEINS

IX. INACTIVATION AND DEGRADATION OF CRYSTALLINE FUMARASE BY BACTERIAL PROTEINASE*

By MINORU INAGAKI, TAKAHIRO MASUDA and KAZUO OKUNUKI

(From the Department of Biology, Faculty of Science, University of Osaka, Osaka)

(Received for publication, April 9, 1958)

In the previous papers of this series (*1–7*), it has been shown that the inactivation of some crystalline enzymes, such as α-amylase of microorganisms, catalase of beef liver, triosephosphate dehydrogenase of rabbit muscle, alcohol dehydrogenase of baker's yeast, and lactic acid dehydrogenase of beef heart muscle, proceeds in parallel with the denaturation of the protein during urea-, heat-, and acid-treatments. No enzyme was digested by bacterial proteinase without previous treatment. It appears to us that these enzymes are usually localized in the soluble portion of the cytoplasm and can be classified as hydrolase and desmolase. We became interested in the problem of the effect of bacterial proteinase on crystalline fumarase obtained from pig heart muscle, because fumarase is an adding enzyme and one of the mitochondrial enzymes.

The present paper shows that, although active fumarase can be crystallized in the native state, after incubation with bacterial proteinase at low temperature and neutral pH the enzymic activity decreases rapidly. To determine whether this inactivation by the proteinase is due only to inhibition, resulting for example from some masking effect, or is due to proteolytic degradation, we have attempted to determine the amino acids and peptides liberated from fumarase by the proteinase. For this the dinitrofluorobenzene method was used. This method is usually used for determination of amino acid end groups. Results were compared with those obtained using the Folin-Ciocalteu method.

The present paper shows that the rapid inactivation of native fumarase by bacterial proteinase is due to proteolytic degradation of the enzyme protein. Unlike the results of previous experiments (*1–7*), it is not possible to distinguish the so-called "ratio of denaturation" of this enzyme from the "ratio of inactivation". It was however possible to demonstrate that the degradation of native fumarase proceeds in parallel with its inactivation.

* An outline of this work was given at the general meeting of the Japanese Biochemical Society at Fukuoka, in October, 1956.

EXPERIMENTAL

Crystalline Fumarase—The enzyme was prepared from pig heart muscle using slight modification of the methods of Massey (*8*) and Alberty (*9*). It was recrystallized twice before use.

Crystalline Bacterial Proteinase—A crystalline bacterial proteinase preparation, "Nagarse" was used, which was supplied by the Nagase Co. Ltd. This was prepared from a culture medium of *B. subtilis* N' by the method of Hagihara (*10*) and was used after recrystallization.

Dinitrofluorobenzene—This compound was synthesized from dinitrochlorobenzene and anhydrous potassium fluoride (*11*).

Assay for Fumarase Activity—Activity was determined by the spectrophotometric methods of Racker (*12*) and Massey (*13*), which measure the disappearance of the double bond of fumaric acid. 0.0167 *M* fumarate was used in these experiments. The rate of the reaction is initially linear and then falls off with decrease in substrate concentration. Therefore initial velocity must be measured. The reaction was initiated by adding the enzyme solution with rapid mixing and the decrease in optical density at 300 mμ was recorded at 15-second intervals. The activity is expressed as the difference between the 30- and 90-second readings. The "per cent inactivation" was calculated from the following equation:

$$\text{Per cent inactivation} = ((\text{Initial activity} - \text{residual activity})/(\text{Initial activity})) \times 100 \quad \text{(i)}$$

Determination of the Course of Digestion of Protein—The method is based on the spectrophotometric measurement of dinitrophenyl derivatives derived from amino acids and peptides liberated during the digestion of the protein.

Two ml. of reaction mixture containing fumarase and proteinase was mixed with 2 ml. of 1.2 *M* trichloroacetic acid and the mixture filtered. Two ml. of the filtrate was neutralized by addition of 4 *M* sodium hydroxide. The neutralized filtrate was introduced into a solution containing 1.0 ml. of saturated sodium bicarbonate, 5 ml. of absolute ethyl alcohol, and 2 ml. of dinitrofluorobenzene solution (1 mg. per ml. alcohol). The mixture was shaken in dark for two hours, and then shaken with 10 ml. of 0.1 per cent sodium bicarbonate solution and 20 ml. of ethyl ether in a separating funel. The water layer was washed with 10 ml. of ether and then neutralized with 2 ml. of 2 *N* hydrochloric acid. The volume of the neutralized solution was adjusted to 20 ml. with distilled water and the adsorption at 350 mμ measured with a spectrophotometer.

Fumarase was completely denatured by heating in a boilng water bath for thirty minutes. After restoration of the original volume, the solution was completely digested by proteinase. It was then mixed with trichloroacetic acid and filtered. The filtrate was treated with dinitrofluorobenzene as described above. The "per cent degradation" was calculated from following equation:

$$\text{Per cent degradation} = ((\text{Optical density of dinitrophenyl derivative in reaction mixture})/(\text{Optical density of dinitrophenyl derivative of completely digested fumarase})) \times 100 \quad \text{(ii)}$$

Determination of Protein Concentration—The concentration of fumarase and bacterial proteinase were determined spectrophotometrically. An optical density at 227 mμ of 1.0 is equivalent to a protein concentration of approximately 2.0 mg. per ml. of fumarase (*14*) and that at 280 mμ is approximately equivalent to 1.0 mg. per ml. of bacterial proteinase.

RESULTS

Inactivation of Fumarase by Bacterial Proteinase—When fumarase was in-

cubated with bacterial proteinase at pH 7.4 (the pH optimum of fumarase) the activity decreased rapidly with time and finally there was complete inactivation (Fig. 1.).

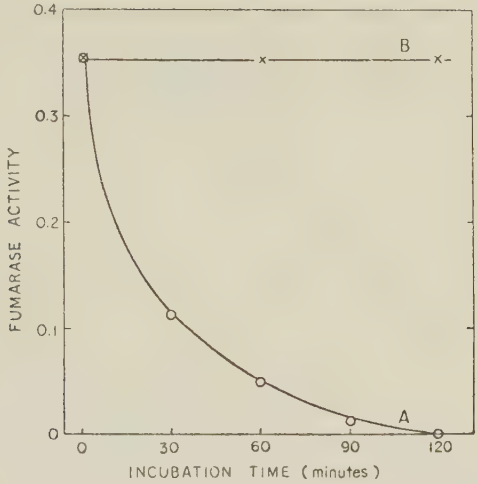


FIG. 1. Inactivation of fumarase by bacterial proteinase.

Curve A: With proteinase. Curve B: Without proteinase. Reaction mixture: Fumarase, 0.015 per cent; bacterial proteinase, 0.003 per cent. *M*/30 phosphate buffer, pH 7.4. Temperature, 30°. Fumarase activity was measured at 25° using 0.3 ml. of 0.167 *M* fumarate, 1.7 ml. of 0.033 *M* phosphate, buffer pH 7.4, and 1 ml. of reaction mixture.

Determination of Amino Acids and Peptides Liberated during Proteolysis—To see whether the inactivation of this enzyme is caused by the proteolytic action of bacterial proteinase or by another type of inhibitory action such as masking, an attempt was made to show liberation of amino acids and peptides from the enzyme protein when incubated with the proteinase.

Two methods are usually used for assay of amino acids and peptides liberated by proteolysis, namely the ninhydrin and Folin-Ciocalteu methods (*15*). The Folin-Ciocalteu reagent is specific for aromatic amino acids and does not react with other amino acids. Ninhydrin is highly sensitive, but reacts with ammonium salts which are present in the enzyme preparation. Inactivation of the enzyme could be caused by liberation of a few amino acids essential for activity. If this is so the above methods are unsuitable. Therefore the dinitrofluorobenzene method, which is usually employed for determination of terminal amino groups (*16*), was used. Fig. 2 shows that there is a linear relationship between the optical density of dinitrophenyl aspartate prepared by the above method, and the concentration of aspartate.

Inactivation and Degradation of Crystalline Fumarase by Bacterial Proteinase—At

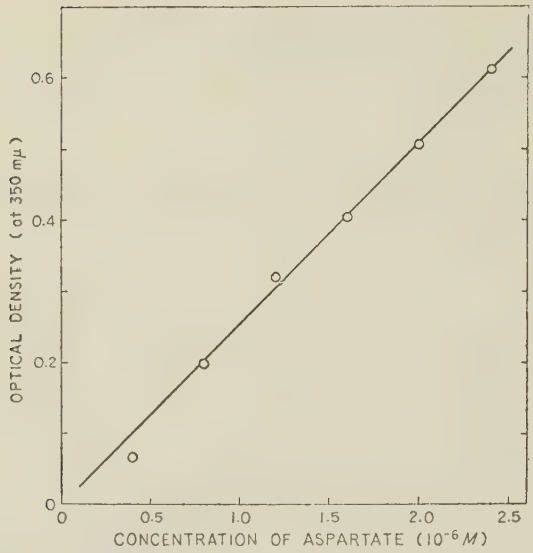


FIG. 2. Correlation of aspartate concentration with formation of its dinitrophenyl derivative.

2 ml. of various concentration of sodium aspartate were treated with dinitrofluorobenzene as described in the text. The concentration of the dinitrophenyl derivative was measured by its absorption at 350 mμ.

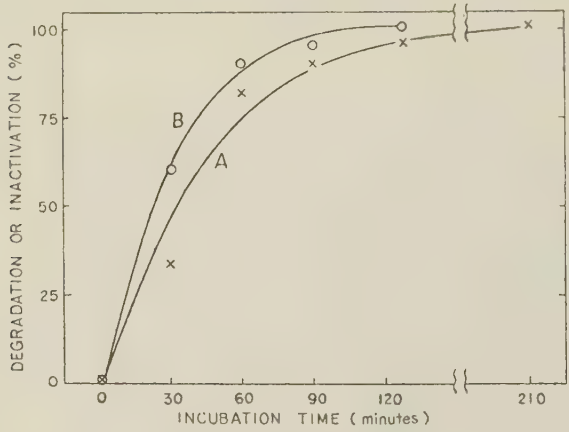


FIG. 3. Time-course of inactivation and degradation of crystalline fumarase by bacterial proteinase.

Reaction mixture: Fumarase, 0.052 per cent; bacterial proteinase, 0.0079 per cent. $M/30$ phosphate buffer, pH 7.4. Temperature 30°. Protein was assayed after mixing 2.0 ml. of the reaction mixture with 2.0 ml. of 1.2 M trichloroacetic acid as described in the text (Curve A). Activity was measured using 0.3 ml. of reaction mixture at 25° (Curve B).

intervals during incubation of the solution with the proteinase, fumarase activity and liberation of amino acids and peptides were measured as described above. Fig. 3 shows that the decrease in fumarase activity was parallel with the liberation of amino acids and peptides. Finally, the activity was completely lost. The amount of amino acids and peptides liberated from the heat denatured fumarase by proteinase was the same as that liberated from enzyme completely inactivated by proteinase. Inactivation of the enzyme is probably caused by the degradation of the enzyme protein.

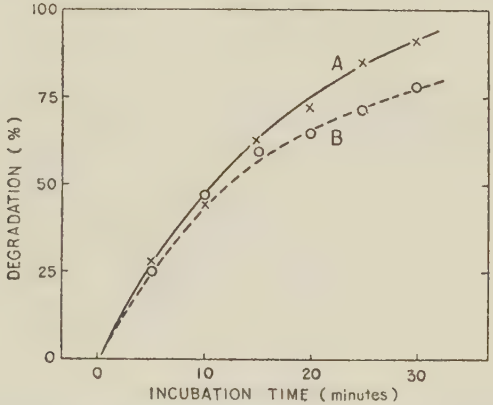


FIG. 4. Comparison of the dinitrofluorobenzene and Folin-Ciocalteu methods of following digestion of casein by bacterial proteinase.

Reaction mixture; casein, 0.1 per cent; proteinase, 0.001 per cent. *M*/30 phosphate buffer, pH 7.4. Temperature, 30°. 2.0 ml. of reaction mixture was added to 2.0 ml. of 1.2 *M* trichloroacetic acid. The mixture was filtered. For assay by Folin-Ciocalteu method 1.0 ml. of the filtrate was neutralized with 5 ml. of 0.45 per cent sodium carbonate and mixed with 1.0 ml. of Folin-Ciocalteu reagent. After 30 minutes at 30°, the color was measured spectrophotometrically at 660 mμ (Curve A). For the assay by dinitrofluorobenzene method 2.0 ml. of the filtrate was used as described in the text (Curve B). The "per cent degradation" of casein was calculated from equation (ii), in which casein completely digested by proteinase was used in place of fumarase.

Comparison of Dinitrofluorobenzene- and the Folin-Ciocalteu Method for Determination of Amino Acids and Peptides by Proteolysis—As described above, the degradation of fumarase protein by the proteinase was demonstrated by the measurement of dinitrophenyl derivatives of liberated amino acids and peptides. To compare the data with results obtained by Folin-Ciocalteu method, casein was used as the protein substrate. The results are shown in Fig. 4. The rate of digestion of casein by bacterial proteinase as estimated by the dinitrofluorobenzene method differed from that by Folin-Ciocalteu

method. The rate of digestion measured by dinitrofluorobenzene method was slower than that by the Folin-Ciocalteu method. The same result was obtained in an experiment on the degradation of fumarase protein (Fig. 5).

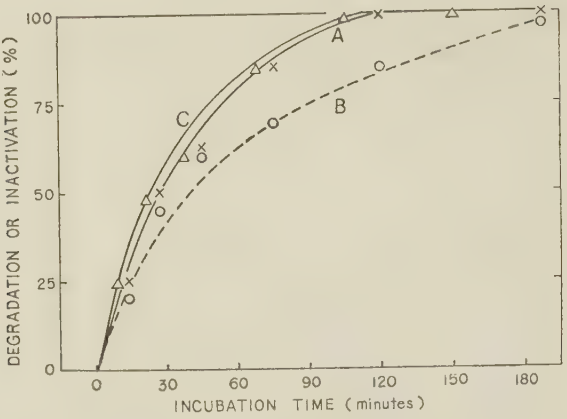


Fig. 5. Comparison of dinitrofluorobenzene and Folin-Ciocalteu methods of following degradation of fumarase by bacterial proteinase.

Reaction mixture: Fumarase, 0.03 per cent; bacterial proteinase, 0.0045 per cent, pH 7.4, in *M*/30 phosphate buffer. Temperature, 30°. 2.0 ml. of reaction mixture was added to 2.0 ml. of 1.2 *M* trichloroacetic acid. The mixture was filtered. The filtrate was used for assay by the Folin-Ciocalteu and dinitrofluorobenzene methods as described in Fig. 4. Curve A: Per cent degradation assayed by Folin-Ciocalteu method. Curve B: Per cent degradation assayed by dinitrofluorobenzene method. Curve C: Per cent inactivation; 0.3 ml. of reactivation mixture was used.

DISCUSSION

It has been assumed that crystalline enzymes with complete activity, generally have a rigid structure and are resistant to digestion by proteinase (*1–7*). Some pretreatment (*e.g.* urea-, heat-, or acid-treatment) was thought to be necessary to make the active crystalline enzymes digestible (*1–7*).

The previous papers (*1–7*) on α-amylase, catalase, triosephosphate dehydrogenase and lactic dehydrogenase support this assumption. However the results reported in this paper provide evidence that certain crystalline enzymes are easily inactivated and digested by bacterial proteinase, without any pretreatment. Also, it may be concluded from results reported here that crystalline enzymes are not necessarily resistant to the digestive action of proteinase. Fumarase was rapidly inactivated by proteinase although the crystalline enzyme had lost very little of its activity after storage for several

months in the refrigerator and in solution retained complete activity at 30° for several hours.

However, it is not yet clear why crystalline fumarase is easily digested by bacterial proteinase. The effect of other proteolytic enzyme and substances on this enzyme will be reported in the next paper.

Finally it should be mentioned that the dinitrofluorobenzene method is suitable for assay of amino acids and peptides liberated from proteins, especially when the sample contains ammonium salts and other than aromatic amino acids.

SUMMARY

Crystalline fumarase is rapidly inactivated by incubation with bacterial proteinase at pH 74. The amino acids and peptides liberated during digestion of the enzyme protein by proteinase were measured using the dinitrofluorobenzene method.

Evidence is given that the inactivation is caused by proteolytic action of the proteinase.

Comparison of the dinitrofluorobenzene and Folin-Ciocalteu methods are also reported.

REFERENCES

(*1*) Okunuki, K., Hagihara, B., Matsubara, H., and Nakayama, T., *J. Biochem.*, **43**, 453 (1956)

(*2*) Hagihara, B., Nakayama, T., Matsubara, H., and Okunuki, K., *J. Biochem.*, **43**, 469 (1956)

(*3*) Hagihara, B., Nakayama, T., Matsubara, H., and Okunuki, K., *J. Biochem.*, **43**, 483 (1956)

(*4*) Hagihara, B., Shibata, T., Sekuzu, I., Hattori, F., Nakayame, T., Nozaki, M., Matsubara, H., and Okunuki, K., *J. Biochem.*, **43**, 495 (1956)

(*5*) Sekuzu, I., Hagihara, B., Hattori, F., Shibata, T., Nozaki, M., and Okunuki, K., *J. Biochem.*, **44**, 587 (1957)

(*6*) Nozaki., M., and Sekuzu, I., Hagihara, B., Yonetani, T., Nakai, T., and Okunuki., K., *J. Biochem.*, **44**, 595 (1957)

(*7*) Sekuzu, I., Yamashita, J., Nozaki, M., Hagihara, B., Yonetani, T., and Okunuki, K., *J. Biochem.*, **44**, 601 (1957)

(*8*) Massey, V., *Biochem. J.*, **51**, 490 (1952)

(*9*) Frieden, C., Bock, R.M., and Alberty, A., *J. Am. Chem. Soc.*, **76**, 2482 (1954)

(*10*) Hagihara, B., *Ann. Rept. Sci. Works, Fac. Sci. Osaka Univ.*, **2**, 35 (1954)

(*11*) Cook, H.G., and Sanders, B.C., *Biochem. J.*, **41**, 558 (1947)

(*12*) Racker, E., *Biochem. et Biophys. Acta*, **4**, 211 (1950)

(*13*) Massey, V., *Biochem. J.*, **53**, 72 (1953)

(*14*) Massey, V., Methods in Enzymology, edited by Colowick, S.P., and Kaplan, N. O., Academic Press Inc., New York, Vol. **I** p. 729 (1955)

(*15*) Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, **73**, 627 (1927)

(*16*) Sanger, F., *Biochem. J.*, **39**, 507 (1945)

The Journal of Biochemistry, Vol. 45, No. 11, 1958

STUDIES ON THE METABOLISM OF GLUCURONIC ACID

COMPARISON OF THE BIOLOGICAL FUNCTIONS OF GLUCURONATE, GLUCURONOLACTONE, AND OF ETHYL GLUCURONATE

By YOHICHI SHIRAI, MASAO WAKABAYASHI, KATSUHISA OHISHI, KAZUKO HAGI, and YOSHINOBU MAYUZUMI

(From the Department of Biochemistry, Kobe Medical College, Kobe)

(Received for publication, April 13, 1958)

It has been elucidated by several authors that glucuronate and glucuronolactone undergo somewhat different metabolic changes in animal body. Packham and Butler (*1*, *2*) have noticed that rats produced more $C^{14}O_2$ from C^{14}-glucuronolactone than from C^{14}-glucuronate. Dutton and Storey (*3*), on the other hand, informed some inhibitory effect of glucuronolactone and no effect of potassium glucuronate on the glucuronide synthesis using rat liver suspenson.

As to the cause of the differences, several facts must be considered. The first is the dissimilarity of the chemical structure of glucuronate and of glucuronolactone, and the second is the disparity in permeability of the both through cell membrane. It may be interesting to determine which of these facts is more important in displaying such metabolic differences mentioned above.

To resolve the problem, the examination of the effect of glucuronic acid ester seems to be important, as the ester is analogous to glucuronate in its structure and as for the permeability, it is supposed to be similar to glucuronolactone because of the absence of ionized group in its molecule. Present paper deals with the comparison of the effects of glucuronate, glucuronolactone, and of ethyl glucuronate on the ascorbic acid biosynthesis in the rat and on the glucuronide synthesis by rat liver slices, and also in the rabbit.

METHODS AND MATERIALS

Glucuronide Synthesis—To measure the glucuronide synthesis by rat liver slices the same method was used as previously reported by Shirai and Ohkubo (*4*). Observation of glucuronide synthesis in the rabbit was conducted as follows. Ten to fourteen male rabbits, weighing about 2 kg., were used in one experiment. The rabbits were divided into two groups (group A and group B). The group A was first injected with 1.0 g. of anthranilic acid subcutaneously and seven days later with 1.0 g. of anthranilic acid and one of the glucuronic acid derivatives simultaneously. The quantity of the latter

compounds given was equimolecular to 1.0 g. of anthranilic acid. With the group B the order of the administration has been reversed, namely, the rabbits were given anthranilic acid and glucuronic acid derivates at first, and anthranilic acid alone later. The administered anthranilic acid had previoutsly been neutralized with sodium carbonate, and the volume of the injected water was fixed at 20 ml. at each time. After the administration of anthranilic acid 24 hour urine was collected under toluene layer, and analyzed for the conjugated anthranilic acid as glucuronide by taking 5 ml. of specimen of the diluted urine (1 : 500). The procedure of the quantitative analysis was practically the same as used *in vitro* test.

Ascorbic Acid Biosynthesis—White rats of either sex, weighing about 100 g. were fed with wheat and water throughout the experiment. After 7 days of preliminary feeding, the rats were separately placed in metallic metabolic cages. Any part of the cages which may contact with rat urine was thickly parafinized. The 24 hour urine was collected in a beaker in which 1 ml. of 20 per cent metaphosphoric acid solution was placed beforehand. After 3 to 5 days of the control period in the metabolic cage, 1 ml. of 0.5 M solution of the samples to be tested were subcutaneously injected to the rats. With respective 24 hour urine, the total ascorbic acid was measured by the method described by Fujita (*5*). The injection was repeated on the next day, followed by 3 days of the second control period. To the control animals was given the same amount of either physiological saline or suitable solutions (see below).

Materials—Glucuronolactone and sodium glucuronate used were the authentic samples and were recrystallized from hot water before use. Ethyl glucuronate was prepared from glucuronolactone and absolute ethanol by the method described by Tsukamoto *et al.* (*6*). The ester obtained was light, colorless long needles. Some samples of the ester contained glucuronolactone as an impurity, but its content was estimated to be far less than 1 per cent by the paper-chromatographic technique (solvent: butanol, acetic acid and water= 4 : 1 : 1, and spraying reagent: 1 per cent aniline hydrochloric acid salt in methanol).

RESULTS

Effects of Glucuronic Acid Derivatives on the Glucuronide Synthesis by Rat Liver Slices—As will be seen in Table I, all the derivatives acted inhibitory when the substances were added to the incubation mixture in higher concentrations (2×10^{-2} M or 10^{-2} M). The degree of inhibitory actions of both glucuronolactone and of ethyl glucuronate were the same and either of them far surpassed that of the glucuronate. In the lower concentration (10^{-3} M), however, the inhibitory action of all the substances diminished and a slight beneficial effect has been observed when ethyl glucuronate was used.

Effects on the Glucuronide Synthesis in the Rabbit—Since the results obtained from group A and B (see general procedures) showed no discrepancy between the two, the data of both groups were put together and treated in the following statistical examination. As indicated in Table II, the effects of glucurolactone and of glucuronate were hardly recognizable. The difference between the values of line "a" and that of line "b" was statistically shown to be meaningless. Ethyl glucuronate, on the other hand, showed remarkable augmenting effect on the conjugation of anthranilic acid as glucuronide, when it was injected to the rabbits together with anthranilic acid. The difference between the values of line "a" and line "b" in this case, was

TABLE I

Effects of Glucuronic Acid Derivatives on the Conjugation of Anthranilic Acid as Glucuronide by Rat Liver Slices

Added glucuronate			Glucuronolactone		Ethyl glucuronate	
Concentration (*M*)	Conjugated (*μg.*)	Increased (+) or decreased (−) (%)	Conjugated (*μg.*)	Increased (+) or decreased (−) (%)	Conjugated (*μg.*)	Increased (+) or decreased (−) (%)
0	9.6	—	9.6	—	9.6	—
2×10^{-2}	8.1	−18.5	4.5	−53.1	4.3	−56.2
0	9.5	—	9.5	—	9.5	—
10^{-2}	8.7	−8.4	6.1	−36.3	6.9	−27.4
0	8.7	—	8.7	—	8.7	—
2×10^{-3}	8.2	−5.7	9.1	+ 4.6	9.4	+ 8.0
0	10.0	—	10.0	—	10.0	—
10^{-3}	8.3	−7.0	10.7	+ 7.0	11.6	+16.0

Note: The numbers of *μg.* in the Table represent the conjugated anthranilic acid per cup.

TABLE II

Effects of Ethyl Glucuronate and of its Related Compounds on the Conjugation of Anthranilic Acid in the Rabbit

	Substances given*	Number of experiment	Anthranilic acid conjugated (*mg.*)
a	Anthranilic acid	10	342.7± 96.7
b	,, +Ethyl glucuronate	10	598.3±142.5
a	Anthranilic acid	14	384.2± 26.8
b	,, +Glucuronolactone	14	363.1± 85.1
a	Anthranilic acid	10	348.4± 89.9
b	,, +Glucuronate	10	339.9± 87.7
a	Anthranilic acid	10	306.5± 88.9
b	,, +Glucose	10	268.6± 70.7
a	Anthranilic acid	10	387.3±142.3
b	,, +Ethanol	10	331.5±121.3

* The dosage of the administered anthranilic acid was 1.0 g. and those of glucuronic acid derivatives and of related compounds were equivalent to the anthranilic acid given.

found to be significant with a reliability of 99.5 per cent or more. To

verify the specificity of the action of ethyl glucuronate, the effects of glucose and of ethanol have been examined. The results, which are shown in Table II, indicate that both substances show no appreciable effect statistically.

Effects on the Ascorbic Acid Biosynthesis in the Rat—As is shown in Table III, the administration of glucuronolactone and of ethyl glucuronate definitely enhanced the excretion of ascorbic acid in urine. Glucuronate, on the other hand, did not show any effect on the ascorbic acid content in urine. To the control animals, 1 ml. of each of the following solutions was injected; 0.9 per cent sodium chloride, 0.5 *M* ethanol, 0.05 *M* glucuronolactone, and 0.005 *M* glucuronolactone. Neither of them showed accerelating effect.

TABLE III

Effects of Glucuronic Acid Derivatives on the Excretion of Ascorbic Acid in Rat Urine

Substances administered*	Number of experiment	Ascorbic acid in daily urine		
		Before the administration (*mg.*)	During the administration (*mg.*)	After the administration (*mg.*)
Glucuronolactone	6	0.453	1.227	0.771
Ethyl glucuronate	6	0.297	1.011	0.358
Glucuronate	6	0.428	0.442	0.416

* The dosage of the administered substances was 1 ml. each of their 0.5 *M* solutions.

DISCUSSION

The previous report from our laboratory (*7*) and that of Storey (*8*) showed that glucuronate strongly inhibited the glucuronide synthesis by rat liver slices. In the present report it is noted that the glucuronate does not seem to exert so much effect compared with glucuronolactone or ethyl glucuronate. The solution of glucuronate previously used was prepared by neutralizing the solution of glucuronolactone by equimolar sodium bicarbonate. The paperchromatogram of the solution has shown that only a little part of the lactone converted to glucuronate under these treatment*. Therefore the inhibitory effect of glucuronate previously noticed should be attributed mostly to glucuronolactone.

In the experiment of the glucuronide synthesis by rat liver slices and in that of ascorbic acid biosynthesis in the rat, glucuronolactone and ethyl glucuronate seemed to behave analogously. Since the content of glucuronolactone in ethyl glucuronate is difinitely less than 1 per cent, and since the reduced amount of glucuronolactone does not show any recognizable

* Unpublished data.

biological effect, the action of ethyl glucuronate should not be regarded as the contamination of glucuronolactone in the ester.

The similarity between the above mentioned biological effects of glucuronolactone and ethyl glucuronate, and the discrepancy between the effects of the both substances and those of sodium glucuronate, may likely indicate that the difference in metabolic fate of glucuronate and of glucuronolactone is mainly due to their imparity in permeability through the cell membrane and not to the ring structural dissimilarity of the both substances.

Finally, ethyl glucuronate enhanced the conjugation of anthranilic acid as glucuronide in the rabbit. From the isotopic studies conducted by Douglas and King (*9, 10*), Packham and Butler (*2*), and Eisenberg *et al.* (*11*), and from the enzymic studies by Dutton and Storey (*3, 12*), Smith and Mills (*13*), and Strominger *et al.* (*14*), it is generally believed that glucuronic acid or α-glucuronic acid-1-phosphate is not a direct precursor of glucuronide*. Therefore the significance of the present results are difficult to interpret. Because of the absence of the accerelating effect of glucose in the present experiment, the effect of ethyl glucuronate may not be due to its conversion into glucose as was considered by Horecker *et al.* (*16*). In connection with the present results, Wakabayashi (*17*) informed that glucuronate accerelated slightly the conjugation of anthranilic acid by the rat liver homogenate.

Südhof's results (*18, 19*), which have shown the enhancing effect of glucose and the inhibitory effect of glucuronolactone on the glucuronide synthesis in the rabbit could not be verified in the present experiment. The conditions of his experiment, however, differ from that of our experiment in several points (dosage, modus of administration, and kind of aglucuron). It is assumed that these differences may have caused such discrepancy in the results.

SUMMARY

1. Some biological effects of glucuronate, glucuronolactone, and of ethyl glucuronate were compared.

2. On the glucuronide synthesis by rat liver slices, and on the ascorbic acid biosynthesis in the rat, the effects of glucuronolactone and of ethyl glucuronate were analogous, and distinctly differed from that of glucuronate.

3. Ethyl glucuronate alone remarkably augmented the conjugation of anthranilic acid in the rabbit, and the other substances tested were without effect.

4. Cause of the differences between the metabolic fate of glucuronolactone and that of glucuronate was discussed.

* It must be mentioned here that the previous report from our laboratory (*15*), which has shown the accerelating effect of α-glucuronic acid-1-phosphate on the conjugation of anthranilic acid as glucuronide by rat liver slices, has been concluded as erroneous after several re-examinations in our laboratory.

The authors wish to express their gratitude to Prof. H. Mabuti for his interest in the present studies. The work was supported by the grant of the Science Research Found of the Ministry of Education.

REFERENCES

(*1*) Packham, M.A., and Butler, G.C., *J. Biol. Chem.*, **207**, 639 (1954)
(*2*) Packham, M.A., and Butler, G.C., *Arch. Biochem. Biophys.*, **56**, 551 (1955)
(*3*) Dutton, G.J., and Storey, I.D.E., *Biochem. J.*, **57**, 275 (1955)
(*4*) Shirai, Y., and Ohkubo, T., *J. Biochem.*, **41**, 337 (1954)
(*5*) Fujita, A., Chemical Estimation of Vitamins (*Japanese*), Nankodo Press, Tokyo, p. 176, (1948)
(*6*) Tsukamoto, H., Hamana, M., Kato, K., and Kuroda., T., *J. Pharmac. Soc. Japan* (*Japanese*) **76**, 1282 (1956)
(*7*) Shirai, Y., and Ohkubo, T., *J. Biochem.*, **41**, 341 (1954)
(*8*) Storey, I.D.E., *Biochem. J.*, **47**, 212 (1950)
(*9*) Douglas, J.E., and King, C.G., *J. Biol. Chem.*, **198**, 187 (1952)
(*10*) Douglas, J.E., and King, C.G., *J. Biol. Chem.*, **203**, 889 (1953)
(*11*) Eisenberg, F., Field, J.B., and Stetten, D.W., *Arch. Biochem. Biophys.*, **59**, 297 (1955)
(*12*) Storey, I.D.E., and Dutton, G.J., *Biochem. J.*, **59**, 279 (1951)
(*13*) Smith, E.E.B., and Mills, G.T., *Biochim. et Biophys. Acta*, **13**, 386 (1954)
(*14*) Strominger, J.L., Kalcker, H.M., Axelrod, J., and Maxwell, E.S., *J. Am. Chem. Soc.*, **76**, 6411 (1954)
(*15*) Shirai, Y., and Ohkubo, T., *J. Biochem.*, **41**, 781 (1954)
(*16*) Horecker, B.L., and Mehler, A.H., *Ann. Rev. Biochem.*, **24**, 244 (1955)
(*17*) Wakabayashi, M., *J. Japan. Biochem. Soc.* (*Japanese*), **28**, 624 (1957)
(*18*) Südhof, H., *Hoppe-Seyler's Z.*, **290**, 72 (1952)
(*19*) Südhof, H., *Hoppe-Seyler's Z.*, **296**, 267 (1954)

The Journal of Biochemistry, Vol. 45, No. 11, 1958

THE EFFECTS OF INHIBITORS ON Fe^{59}-INCORPORATION INTO HEME BY DUCK HEMOLYSATE

By SHIGEKI MINAKAMI, YOSHIKO KAGAWA, SEIEI TANAKA, YOSHIKI SUGITA and YOSHIMASA YONEYAMA

(From the Department of Physiological Chemistry and Nutrition, Faculty of Medicine, University of Tokyo, Tokyo)

(Received for publication, May 8, 1958)

The hemolysate of the nucleated avian red cells have been known to have the activity to incorporate radioactive iron into heme (*1*, *2*). The conditions for the incorporation in chicken hemolysate were studied extensively by Goldberg *et al.* (*2*). The intermediates in heme biosynthesis, including glycine, δ-aminolevulinic acid, porphobilinogen and protoporphyrin, have an accelerating effect on the incorporation of radioactive iron. The effect of malonate and lead acetate on the porphyrin biosynthesis and the iron insertion was discussed by them. The use of hemolysate has an advantage in that, besides the insertion of iron into protoporphyrin, the synthesis of protoporphyrin from the intermediates can be observed using radioactive iron as a tracer.

In the previous communication from this laboratory, the effects of some inhibitors on the iron incorporation by canine reticulocytes were reported (*3*). In the present paper, observations on the effects of some inhibitors, such as fluoride, cyanide, 2, 4-dinitrophenol and lead acetate on the heme synthesis by duck hemolysate from the intermediates are presented and the sites of action are discussed.

MATERIALS AND METHODS

Duck Erythrocytes—The blood used was taken from normal adult ducks pooled for marketing, by cutting jugular vein. Approximately 500 ml. of the blood were received into a bottle containing 15 mg. of penicillin G, 15 mg. of streptomycin and 10 mg. of heparin.

Preparation of Hemolysate—The blood was centrifuged and the plasma discarded. The cells were washed twice with cold isotonic potassium chloride solution. The leucocytes were removed by suction. The red cells were hemolysed by addition of two volumes of distilled water and allowed to stand for 5 minutes in an ice bath with occasional stirring. One sixth volume (to the volume of hemolysate) of 0.15 *M* tris (trishydroxymethylaminomethane) buffer, pH 7.5, containing 0.53 *M* potassium chloride was added.

Incubation—Seven milliliters of the hemolysate which corresponded to 2 ml. of the

red cells, were placed in a 50 ml. conical flask. One milliliter of radioactive iron (approximately 10^5 c.p.m.) containing 2 μg. of carrier iron in the form of ferric ammonium citrate, and the substrate (glycine, δ-aminolevulinic acid or protoporphyrin) in 1 ml. of isotonic potassium chloride solution were added. The flasks were incubated for 1.5 hours with gentle shaking, 40–50 per minute, at 37°.

Isolation and Purification of Hemin—At the end of the incubation, the mixture was poured into a 50 ml. centrifuge tube containing 20 ml. of acetone-acetic acid (4 : 1) containing small amount of hydrochloric acid (one drop of conc. HCl per 100 ml. of acetone) by stirring with a glass rod. After standing for ten minutes the mixture was centrifuged and the extract was transferred to a distillation flask.

A few milligrams of sodium chloride and boiling stone were introduced into the flask and acetone was distilled completely off by heating in a boiling water bath. The hemin crystallized was collected by centrifugation and washed two times with diluted hydrochloric acid.

This is a modification of the method established by Chu and Chu (*4*) in adaptation to a large number of samples to be disposed in a short time. The procedure has certain advantages compared to the generally used Fischer's method (*5*), in that hemin crystallizes out in good yield from a dilute solution of hemoglobin and further recrystallization is unnecessary to remove the contamination with radioactive non-hemin iron.

Determination of Specific Activity—The hemin crystal was dissolved in one drop of concentrated sodium hydroxide, washed into a test tube with 1 ml. of water and the radioactivity was determined by means of well-type scintillation counter (Scientific Research Institute, Tokyo).

The solution was then diluted to 20 ml., and an aliquot of 0.3 ml. was pipetted into a colorimetric tube and after addition of few drops of 10 per cent potassium cyanide solution, made up to 5 ml. with water. The optical density of cyanide-hemochromogen at 540 mμ was read with the aid of Klett-Summerson photoelectric colorimeter. Specific activity was expressed as c.p.m. per mg. of hemin.

Amount of the Iron Incorporated into Hemin—The rate of heme synthesis was calculated from the per cent uptake of radioactive iron added to the medium. Hemoglobin content in the hemolysate was determined colorimetrically as oxyhemoglobin.

Substrate—Protoporphyrin was prepared by acid hydrolysis of crystalline methyl ester by the method of Grinstein (*6*). The porphyrin was dissolved in a minimum amount of diluted ammonia water and diluted with isotonic solution of potassium chloride to the desired volume. δ-Aminolevulinic acid was commercially provided*. The final concentrations of the substrates were as follows unless otherwise stated: glycine, 5×10^{-2} *M*; δ-aminolevulinic acid, 1×10^{-4} *M*; protoporphyrin, 5×10^{-5} *M*.

RESULTS AND DISCUSSION

Properties of Duck Hemolysate as a Heme Forming System—The time course of the iron incorporation using glycine as a substrate is shown in Fig. 1. Almost linear increase of the count per mg. of hemin was observed for the first 1.5 hours. Similar results were obtained when δ-aminolevulinic acid and protoporphyrin were added as substrates.

The influence of the concentrations of the substrates on heme synthesis

* Synthetic, Daiichi Pure Chemicals, Co. Ltd., Tokyo. The authors acknowledge the gift of the sample to Daiichi Pure Chemicals, Co. Ltd.

was studied, with the results similar to that reported by Goldberg *et al.* (*2*), that is, the uptake from 2 μg. of iron attained to its maximum at the concentrations of the substrates; glycine, 5×10^{-2} *M*; δ-aminolevulinic acid, 1×10^{-4} *M*; protoporphyrin, 5×10^{-5} *M*. The relation of the iron incorporation to the glycine concentration is illustrated in Fig. 2 as an example.

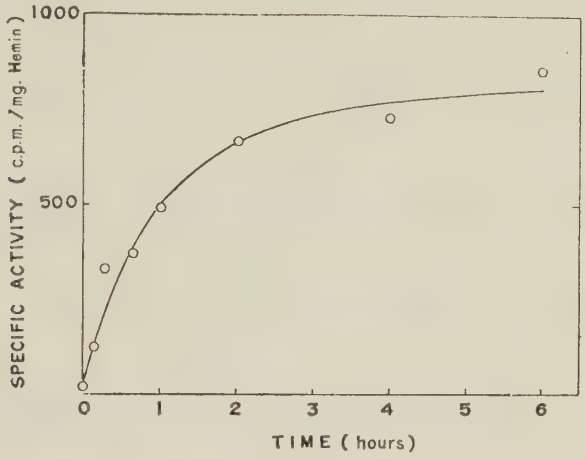


FIG. 1. Time course of the iron incorporation using glycine as the substrate. The reaction mixture 10 ml. contained 7 ml. of hemolysate, 2 μg. of radioactive iron, and glycine to give a final concentration of 5×10^{-2} *M*.

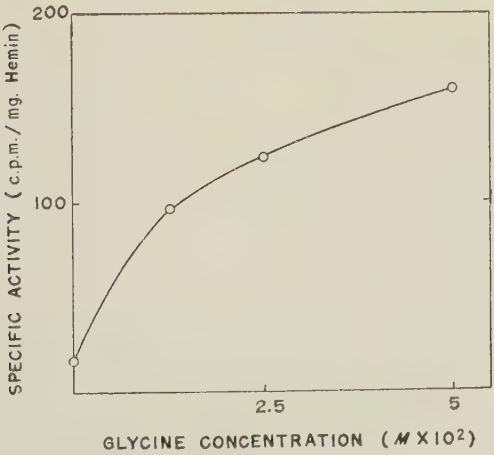


FIG. 2. Acceleration of the iron uptake by the addition of different concentrations of glycine. Conditions other than the concentration of glycine were the same as in Fig. 1. Incubation time, 1.5 hours.

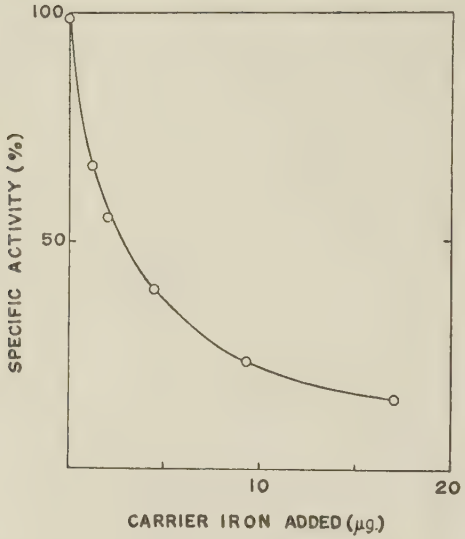


FIG. 3. The effect of iron concentrations of the iron incorporation. Conditions other than the amount of added carrier iron were the same as in Fig. 1. Incubation, 1.5 hours.

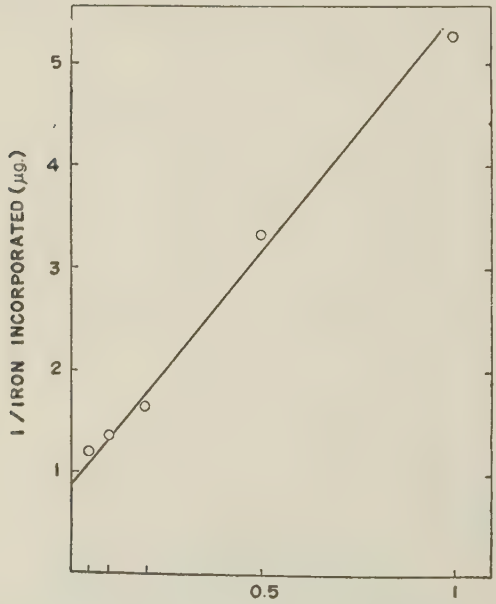


FIG. 4. Double reciprocal relation of the iron incorporation and the amount of carrier iron added.

The addition of carrier iron caused a decrease in iron uptake as shown in Fig. 3. The double reciprocal relation between the iron concentration and the amount of the radioactive iron incorporated is plotted from the data (Fig. 4). Linear relation may be explained either by Michaelis-Menten's hypothesis or by the existence of the endogeneous iron pools available for the iron incorporation into heme. The amount of heme synthesized by 1.5 hour incubation with the hemolysate corresponding to 2 ml. of duck erythrocytes was calculated to be 10 μg. The capacity of normal duck erythrocytes to synthesize heme has been calculated by Jensen *et al.* (*7*) to be approximately 936 μg. of heme in 100 ml. of normal duck blood with packed red cell volume of 42 ml./dl., 6 per cent reticulocytes and a plasma iron concentration of 180 μg. per cent.

Influence of Inhibitors on the Iron Uptake—As shown in Fig. 5, cyanide inhibited the heme synthesis from glycine, δ-aminolevulinic acid and protoporphyrin in varying degree, effecting more intensively when the first one

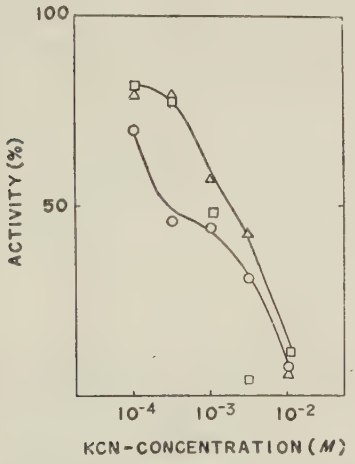


Fig. 5. Influence of cyanide on heme synthesis (conditions as in text). The following substrates were used: —○— glycine, —△— δ-aminolevulinic acid, —□— protoporphyrin.

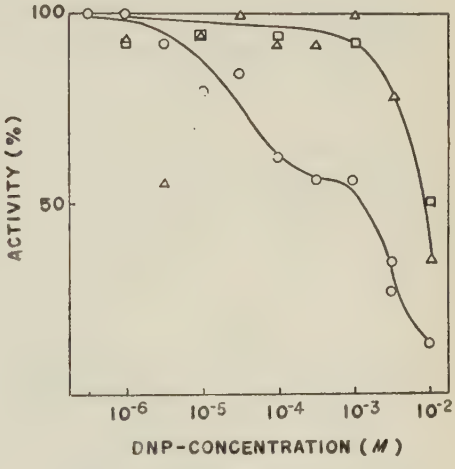


Fig. 6. Influence of 2,4-dinitrophenol on heme synthesis (conditions as in text). Substrates; —○— glycine, —△— δ-aminolevulinic acid, —□— protoporphyrin.

was added. Thus, with 3×10^{-4} *M* cyanide, where the heme biosynthesis from δ-aminolevulinic acid or protoporphyrin was reduced by only about 20 per cent, the heme synthesis from glycine was inhibited more than 50 per cent. The curve, representing the relation between the inhibitor concentrations and the activity, showed a sigmoid curve when δ-aminolevulinic acid or protoporphyrin was used as the substrate. The inhibition curve, obtained with glycine may be regarded as a combination of two sigmoid curves, one from the glycine added and the other from the intermediates of endogeneous origin. It seems clear, therefore, that in this system cyanide inhibits the biosynthesis of heme

at two points, *i.e.* the formation of δ-aminolevulinic acid from glycine and the insertion of iron into protoporphyrin.

Inhibition by 2, 4-Dinitrophenol—A similar inhibition was observed when 2, 4-dinitrophenol was added to the medium (Fig. 6). The decrease in the heme biosynthesis from glycine by the addition of 2, 4-dinitrophenol at lower concentrations, may be attributed to the inhibition at the point prior to the formation of δ-aminolevulinic acid. The Fe^{59}-incorporation observed in the presence of δ-aminolevulinic acid and protoporphyrin was also inhibited by 2, 4-dinitrophenol, but only at higher concentration. The requirement of energy supply in the heme synthesis especially at the intermediary steps between glycine and δ-aminolevulinic acid is suggested.

Inhibition by Lead—The observation of Goldberg and others (*2*) on the inhibition of the heme synthesis by lead acetate was confirmed (Fig. 7). They indicated the possibility that lead inhibits the biosynthesis of heme at least at

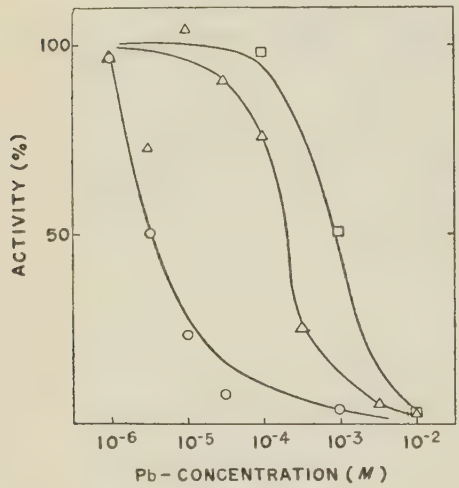


FIG. 7. Influence of lead acetate on heme synthesis (conditions an in text). Substrates; —○— glycine, —△— δ-aminolevulinic acid, —□— protoporphyrin.

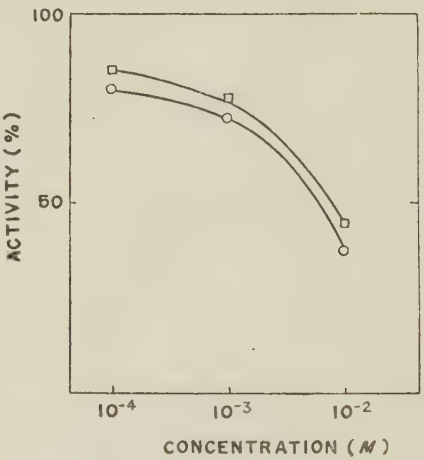


FIG. 8. Influence of sodium floride on heme synthesis (conditions as in text). Substrates; —○— glycine, —□— protoporphyrin.

two places: prior to the formation of δ-aminolevulinic acid and at the stage of the conversion of protoporphyrin to heme. Dresel and Falk have expressed their opinion from the experiment of porphyrin formation that lead inhibits some step in the synthesis of a precursor of heme, rather than the incorporation of iron into the porphyrin nucleus (*8*). However, it may be concluded from the present experiment that lead specifically inhibits both of the porphyrin biosynthesis and the iron insertion.

Inhibition by Fluoride—The heme synthesis from glycine and protoporphyrin was inhibited to the same extent by fluoride as shown in Fig. 8. The effect may be due to the inhibition of iron insertion into porphyrin.

Comparison with Intact Canine Reticulocytes—The incorporation of iron into heme in canine reticulocytes was inhibited by various inhibitors as shown in the previous report (*3*). The concentrations exhibiting 50 per cent inhibition were as follows: potassium cyanide, $10^{-3.5}$ *M*; sodium fluoride, $10^{-2.5}$ *M*; 2, 4-dinitrophenol, $10^{-4.5}$ *M*; lead acetate, $10^{-5.5}$ *M*. These values agree with the concentrations of the inhibitors for 50 per cent inhibition of heme synthesis from glycine. The heme synthesis from δ-aminolevulinic acid or protoporphyrin showed different attitude.

SUMMARY

1. The properties of duck hemolysate as a heme forming system from glycine, δ-aminolevulinic acid or protoporphyrin were investigated.

2. The influence of inhibitors on the heme synthesis from the different intermediates was studied to characterize the nature of respective steps of heme formation. The iron uptake into heme by the addition of glycine was inhibited by cyanide, 2, 4-dinitrophenol or lead acetate at lower concentrations, whereas the biosynthesis from δ-aminolevulinic acid or protoporphyrin needed higher concentrations for inhibition. Fluoride inhibited the iron uptake at the same rate either with glycine or protoporphyrin.

The authors express their cordial thanks to Prof. H. Yoshikawa for his interest during this work. Thanks are also due to the students of this faculty, Mr. H. Itakura, N. Kaihara, Y. Kanazawa, N. Kato, H. Onda and E. Ozawa for their cooperations.

REFERENCES

(*1*) Granick, S., *Fed. Proc.*, **13**, 319 (1954)
(*2*) Goldberg, A., Ashenbrucker, H., Cartwright, G.E., and Wintrobe, M.M., *Blood*, **9**, 821 (1956)
(*3*) Yoshiba, A., Nakao, K., Minakami, S., and Yoneyama, Y., *J. Biochem.*, **45**, 921 (1958)
(*4*) Chu, T.C., and Chu, E.J., *J. Biol. Chem.*, **212**, 1 (1955)
(*5*) Fischer, H., *Organic syntheses*, **21**, 53 (1941)
(*6*) Grinstein, M., *J. Biol. Chem.*, **167**, 515 (1947)
(*7*) Jensen, W.N., Ashenbrucker, H., Cartwright, G.E., and Wintrobe, M.M., *J. Lab. Clin. Med.*, **42**, 833 (1953)
(*8*) Dresel, E.I.B., and Falk, J.E., *Biochem. J.*, **63**, 72 (1956)

The Journal of Biochemistry, of Vol. 45, No. 11, 1958

EFFFCT OF PRE-ILLUMINATION UPON THE HILL REACTION IN CHLORELLA CELLS

By TETSUYA KATOH, TOYOYASU HIROKAWA and SHIGETOH MIYACHI

(*From the Department of Biochemistry and Biophysics, and the Institute of Applied Microbiology, University of Tokyo, Tokyo*)

(Received for publication, July 9, 1958)

Using radioactive carbon as a tracer Calvin and Benson (*1*) discovered that the capacity of green cells for fixing CO_2 in the dark was greatly enhanced when the cells were pre-illuminated immediately prior to the provision of CO_2. The photogenic agent responsible for the enhancement of CO_2-fixation was found to be a labile substance, being able to survive only a few minutes after the cessation of pre-illumination. In previous reports from this laboratory (*2–5*) it was shown, also using $C^{14}O_2$ as a tracer, that the photogenic agent in question has a property of reacting with, and consumed by, oxidizing agents such as oxygen, quinone and H_2O_2, indicating that the agent is a reducing substance functioning, both in the Hill reaction and in the process of CO_2-fixation (photosynthesis). The process of decay of the agent occurring in the dark was interpreted as being due to the reaction between the agent and the oxygen which was assumed to be generated in the process of photochemical formation of the agent from its precursor. If the agent is involved in the mechanism of the Hill reaction, its behavior must be followed by appilcation of the Hill reagents instead of $C^{14}O_2$, and the Hill reaction must be brought about *in the dark* when the cells have been pre-illuminated immediately prior to the provision of the exogenous oxidant.

Using spinach chloroplasts as the material and 2,6-dichlorophenolindophenol (DPIP) as the oxidant, Mehler (*6*) reported, however, that no increase was observed in the dark reduction of the dye after pre-illumination, and based on this observation he assumed that the agent responsible for the CO_2-fixation after pre-illumination might be a CO_2-acceptor rather than a reducing substance. In disagreement with this, Krasnovsky and Kosobutskaya (*7*) observed, employing a "green solution" from bean leaf and DPIP and several other redox dyes as oxidants, that the reduction of the dyes was greatly accelerated by the effect of pre-illumination.

The experiment with intact *Chlorella* cells now to be described will show that the reduction of CPIP in the dark is actually enhanced by pre-illumination and that the process of photochemical formation of the reducing agent as well as its decay in the dark, which has hitherto been investigated always

with $C^{14}O_2$ as a traces, can be followed successfully by measuring the quantily of DPIP reduced by the reducing agent. It was concluded that the negative result reported by Mehler was due to the insufficiency of the time of pre-illumination applied in his experiment.

MATERIAL AND METHOD

The material used were the "dark cells" of *Chlorella ellipsoidea* (*8*), which were suspended in 0.02 *M* phosphate buffer of pH 6.8. The substance chosen as the oxidant was DPIP, the concentration of which was determined by measuring the optical density at 610 mμ. Experimental vessel used was the same in principle as Apparatus I which was described in one of our earlier papers (*2*). It consisted of a round flat vessel (lollipop) and an exchangeable dark vessel connected to it. The algal suspension was placed in the flat vessel while the dark vessel containd 2.0 ml. of ethyleneglycol (see below). The cell suspension was illuminated from both sides,* or kept dark, with constant bubbling of N_2, and after a certain period of time—and after turning off the light in the case of preillumination experiments –the dye solution (oxidized form; final concentration: $4–8\times10^{-5}M$) was injected anaerobically into the algal suspension, and at intervals aliquots (7.5 ml.) were swiftly transferred into the dark vessels. Immediately after mixing the suspension with ethyleneglycol (final concentration: 20 per cent) in the dark vessel, the latter was detached from the flat vessel and cooled down instantly to a temperature lower than 0°. The purpose of using ethyleneglycol was to avoid freezing at the low temperature which was applied to stop further reduction of the dye caused by algal enzymes. Cooled samples were then centrifuged using a refrigerating centrifuge, and the dye solution, freed from algal cells and divided into two portions, was subjected to the measurement of optical density. With one portion of the solution the quantity of remaining oxidized dye was directly measured, and with the other the total quantity of the dye was measured after treating the solution with a sufficient quantity of potassium ferricyanide; the quantity of the reduced dye was calculated from the difference of the two readings. In the non-pre-illuminating experiment, the dye solution was added to the cell suspension after the algae had been kept with dark constant bubbling of N_2, whic h then was followed by the same treatment as in the illuminating experiment.

RESULTS

Effect of Pre-Illumination on the Dark Reduction of DPIP—Fig. 1 shows the time courses of dye reduction in the dark by the cells which had been pre-illuminated for a sufficient length of time (40 minues) and by the cells which had been kept dark during the same period. As may be seen from the figure, the course of the reduction of DPIP by non-pre-illuminated cells showed a steady linear ascension, whereas the reduction effected by pre-illuminated cells displayed an almost instant upswing to a certain level, which was followed by a gradual ascension with a tangent that was virtually the same as that of the curve obtained with non-pre-illuminated cells. The vertical distance between the two parallel curves, or the intersection with the ordinate obtained by extrapolation of the upper curve may be regarded as

* Light intensity applied was $2\times10{,}000$ lux.

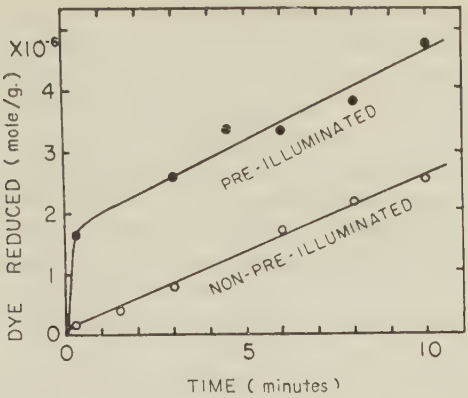


Fig. 1. Time courses of dye-reduction in the dark by sufficiently pre-illuminated and non-per-illuminated algal cells (Density of *Chlorella*: 0.6 ml./100 ml., Temperature: 10°). Ordinate: the amount of DPIP reduced by *Chlorella* cells; Abscissa: the time of contact of cells with DPIP in the dark.

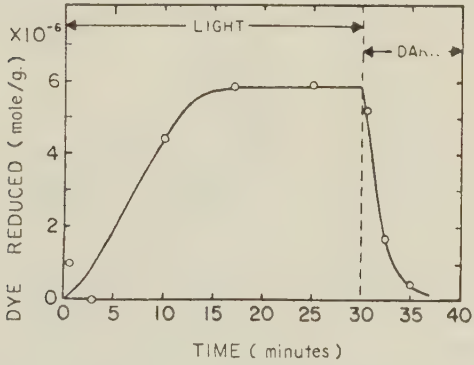


Fig. 2. Formation of light-induced reducing power in the light and its decay after the light was turned off (Density of *Chlorella*: 1.2 ml./100 ml., Temperature: 15°).

Ordinate: the amount of DPIP reduced by the effect of pre-illumination: Abscissa: the time which the cells spent in the light and dark, as indicated, prior to the provision of DPIP (Before the beginning of illumination, cells were kept dark for 30 minutes with constant bubbling of N_2 to make them adapt to the darkness).

showing the quantity of the reducing agent produced by the effect of pre-illumination.

By adopting the extrapolation method, the light induced capacity for reduction of DPIP was measured as a function of the time of pre-illumination

The results obtained are reproduced in Fig. 2. It is clear from the figure that the capacity for the reduction of DPIP increased with the duration of pre-illumination and attained a stationay level about 10–20 minutes after the beginning of illumination. When at the stage the light was turned off, the capacity decreased rapidly with a half life of about 2 minutes. All these phenomena are in accordance with the behavior of the reducing agent—designated by *R* in our previous papers (*2–5*)—which was investigated by using $C^{14}O_2$ as a tracer. It should be remarked, however, that, unlike the *R*-level measured with $C^{14}O_2$, reducing capacity showed a tendency of gradual decrease on prolonged illumination, and the highest level attained fluctuated from sample to sample; the reasons for these phenomena are not yet clear at present.

Effect of DPIP on the Decay of Light-Induced CO_2-Fixing Capacity in the Dark—By using C^{14} as a tracer, it has been observed (*2–4*, *8*) that the decay of the reducing agent (*R*) in the dark was markedly accelerated by the presence of oxygen, H_2O_2 or quinone. To investigate whether the same

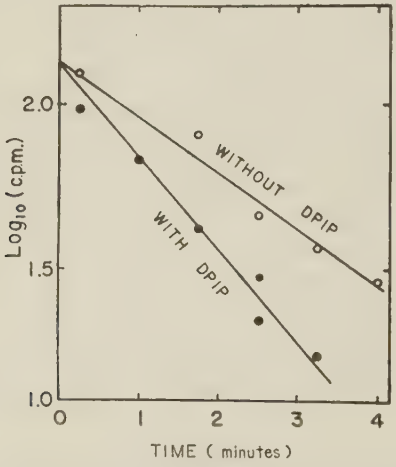


Fig. 3. Semi-logarithmic plot of the process of decay in the dark of light-induced $C^{14}O_2$-fixing power in the presence and absence of DPIP ($2.5\times10^{-5}M$).

Ordinate: c.p.m. of C^{14} fixed in 10 seconds in the dark; Abscissa: the time which the cells spent after the light was turned off, with or without the addition of DPIP, as indicated, before contact with $C^{14}O_2$ in the dark.

effect be brought about by DPIP, the algae were first illuminated for 40 minutes in N_2 atmosphere, then DPIP (final concentration: $2.5\times10^{-5}M$) was added simultaneously with turning off the light, and the time course of the decay of photo-induced CO_2-fixing power was followed by measuring the amount of $C^{14}O_2$ fixed in 10 seconds in the dark. Fig. 3 shows a semilogarithmic plot of the data obtained. The marked increase of the tangent of the

curve caused by the dye indicates that the reducing agent R was consumed by DPIP.

DISCUSSION

From the results represented above it is clear that upon illumination of algal cells some reducing substance responsible for the reduction of DPIP was produced. This finding is contradictory to the observation of Mehler (*6*) who, using isolated chloroplast, could not demonstrate the after-effect of pre-illumination to provoke the reduction of DPIP. It should be pointed out, however, that in his experiment the pre-illumination lested only 1/2 to 2 minutes, which, according to our observation, was such a short time as to produce only a minute fraction of the stationary level of the photogenic reducing agent. Conceivably, the insufficiency of the length of pre-illumination was the cause of the negative result obtained in Mehler's experiment. The fact that the processes of the formation of the reducing substance upon illumination and of its decay upod cessation of illumination was quite similar to the corresponding processes of photogenic agent(s) (R) measured with $C^{14}O_2$, points strongly to the identity of these two agents. It may be ascertained that the substance R, although its chemical nature is not clear at present, is a reducing agent functioning both in the normal photosynthesis and in the Hill reaction.

SUMMARY

1. Using intact *Chlorella* cells, the effect of pre-illumination upon the dark reduction of 2,6-dichlorophenolindophenol (DPIP) was investigated.

2. It was found that the dark reduction of DPIP was greatly enhanced by pre-illumination, indicating that the reducing agent functioning in the Hill reaction is produced in the light and subsequently reacts with the Hill reagent. Using the degree of reduction of DPIP in the dark as a measure of the level of the reducing agent, the courses of its formation in the light and of its decay in the dark were followed. The close similarity observed between the courses thus followed and those investigated by using $C^{14}O_2$ as a tracer (according to Calvin and Benson) showed that the substance investigated by the two different methods was one and the same agent having a reducing power.

This work was carried out under the direction of Prof. H. Tamiya, to whow we are indebted for constant guidance in the course of the work. Thanks are also due to Prof. A. Takamiya for valuable advice. Financial aid from the Ministry of Education is gratefully acknowledged here.

REFERENCES

(*1*) Calvin, M., and Benson, A.A., *Science*, **107**, 476 (1948)
(*2*) Miyachi, S., Izawa, S., and Tamiya, H., *J. Biochem.*, **47**, 221 (1955)

(3) Miyachi, S., Hirokawa, T., and Tamiya, H., *J. Biochem.*, **42**, 737 (1955)
(4) Tamiya, H., Miyachi, S., and Hirokawa, T., "Some New Preillumination Experiment with Carbon-14" in Research in Photosynthesis, Ed. Gaffron, H. *et al.*, Interscience Publishers, Inc., New York, p. 213 (1957)
(5) Hirokawa, T., Miyachi, S., and Tamiya, H., *J. Biochem.* in press.
(6) Mehler, A.H., *Arch. Biochem. Biophys.*, **33**, 65 (1951)
(7) Krasnovsky, A.A., and Kosobutskaya, L.M., *Doklady Akad. Nauk. S.S.S.R.*, **82**, 761 (1962)
(8) Tamiya, H., Iwamura, T., Shibata, K., Hase, E., and Nihei, T., *Biochem. et Biophys. Acta*, **12**, 23 (1953)

The Journal of Biochemistry, Vol. 45, No. 11, 1958

THE EFFECTS OF INHIBITORS ON THE SYNTHESIS OF HEMOGLOBIN IN CANINE RETICULOCYTES*

By AKIRA YOSHIBA, KIKU NAKAO**

(*From the Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Tokyo*)

SHIGEKI MINAKAMI and YOSHIMASA YONEYAMA

(*From the Department of Physiological Chemistry and Nutrition, Faculty of Medicine, University of Tokyo, Tokyo*)

(Received for publication, May 22, 1958)

The ability of reticulocytes to synthesize hemoglobin *in vitro*, has been demonstrated with the aid of C^{14}-labelled amino acids (*1*, *2*) or radioactive iron (*3*) as a tracer. The insertion of iron into heme might be the final stage of heme synthesis, while there has been a good reason to believe that the synthesis of heme and globin is so subtly controlled (*4*), that the overproduction of neither heme nor globin is found in ordinary conditions. Thus the incorporation of iron into heme can be used not only for the indices of heme synthesis but also for hemoglobin synthesis as a whole. From this standpoint, studies on the influence of some inhibitors upon the incorporation of radioactive iron into heme in canine reticulocytes were carried out.

MATERIALS AND METHODS

Preparation of Reticulocytes—Phenylhydrazine or acetylphenylhydrazine was injected subcutaneously on alternate days to adult dogs. After considerable reticulocytosis appeared about a week after the first injection, 120–150 ml. of the blood was drawn from the cubital vein into a syringe containing heparin as an anticoagulant. The plasma and leukocytes layer were discarded after being separated by centrifugation. The upper part of the erythrocytes layer, rich in reticulocytes was resuspended into the plasma with the final hematocrit value of 45 per cent.

Incubation—An aliquot of the radioactive iron solution containing 1–2 μc of Fe^{59} and 1–2 μg. of carrier iron in the form of ferric chloride, was added into the red cells suspension prepared as above mentioned. Nine milliliters of this reaction mixture was taken into a 50 ml. conical flask to which was added 1 ml. of physiological saline containing a proper amount of inhibitor. The flasks were shaken in a thermostat at 37° for 3 hours. The erythrocytes were separated from the reaction mixture by centrifugation at the end of a 3 hours' incubation. Both of the cells and the plasma

* Preliminary report of this paper has already been published in Japanese (*9*).

** Present Address; Department of Internal Medicine, Faculty of Medicine, Gumma University, Maebashi.

was subjected to the following analytical procedures.

Crystallization and Purification of Hemin –The erythrocytes were washed three times with physiological saline to prepare hemin crystals according to Fischer. The crystals obtained were washed several times with 1 per cent hydrochloric acid. Sometimes the repetition of gentle centrifugation and decantation is necessary to remove denatured proteins overlying upon the heavy crystals of hemin.

Measurement of Radioactivity –Hemin crystals and plasma were subjected to wet ashing with sulfuric acid and perchloric acid, neutralized by adding concentrated ammonia water and the aliquot was removed and used for iron determination. The remainder was precipitated by adding ammonium polisulfide and measurement of radioactivity was carried out on the precipitate with the aid of a Geiger-Muller counter (*5*).

Determination of Iron and Hemoglobin—Iron was determined colorimetrically using *o*-phenanthroline. The hemoglobin content of the red cell suspension was determined by the cyanmethemoglobin method with the aid of a Klett-Summerson photoelectric colorimeter at wavelength 540 mμ.

Specific Activity and Total Activity—The specific activity of hemin was expressed as c.p.m. per mg. of hemin iron. The total radioactivity of hemin was obtained by the specific activity of hemin multiplied by its content in the incubation mixture. The specific and the total radioactivities of the plasma were obtained in the same way. Amount of non-hemin iron in erythrocytes was computed from the difference between the total radioactivity added to the reaction mixture and the sum of the total radioactivities found in hemin and plasma.

RESULTS AND DISCUSSION

Hemin Iron and Non-Hemin Iron—The increase in iron incorporation into heme, the decrease of plasma iron and the increase of non-hemin iron during incubation were as shown in Fig. 1. Nakao (*6*) regarded the non-hemin iron in erythrocytes as a precursor for heme synthesis whereas Jensen *et al.* (*7*) explained the penetration of iron by mere diffusion. The significance of non-hemin iron present in erythrocytes is, however, difficult to explain, as there is no reliable direct method for its estimation.

Inhibitor Experiments—The inhibition of hemoglobin synthesis by various inhibitors was shown in Table I. In cases of fluoride, fluoroacetate 2,4-dinitrophenol and lead acetate, the effects as the various concentrations were examined (Fig. 2).

To sum up the effect of these inhibitors, the action of chelating agent such as *o*-phenanthroline and diethyldithiocarbamate may attributed to their combination with iron. The similar explanation may be possible in cases of cyanide, atebrin and fluoride. However, the effect of metabolic inhibitors such as fluoroacetate and 2,4-dinitrophenol could not be explained as the direct inhibition upon iron incorporation. In the latter cases, the inhibition of energy production and utilization may have caused a decreased formation of heme or globin precursors, followed by decreased iron incorporation. The effects of several metabolic inhibitors on the incorporation of C^{14}-labelled amino acids in rabbit reticulocytes was discussed by Borsook *et al.* (*7*). The similar inhibitory actions of some agents upon the protoporphyrin and

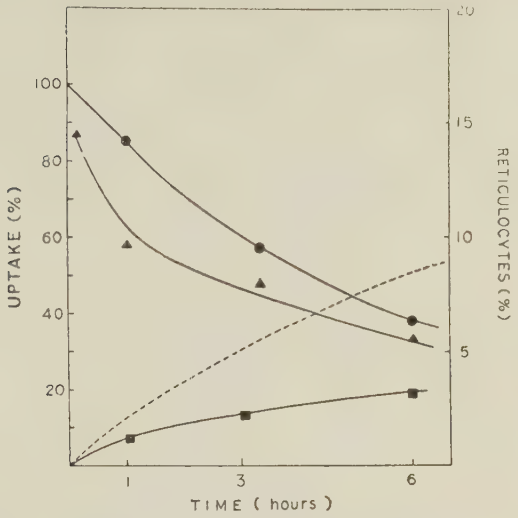


FIG. 1. Time course of iron uptake into heme. —■— heme iron, —●— plasma iron, - - - - - non-hemin iron, —▲— number of reticulocytes in 100 erythrocytes.

TABLE I

Influence of Inhibitors on the Iron Incorporation into Heme in Canine Reticulocytes

Inhibitors	Incorporation (expressed as per cent of control)
Control	100
Cyanide ($10^{-3}M$)	0
Fluoroacetate ($10^{-3}M$)	0
Monojodoacetate ($10^{-3}M$)	42
Fluoride ($5\times10^{-3}M$)	41
2,4-Dinitrophenol ($10^{-3}M$)	13
Thyroxine ($10^{-5}M$)	100
o-Phenanthroline ($10^{-3}M$)	0
Diethyldithiocarbamate ($10^{-2}M$)	43
Atebrin ($10^{-2}M$)	0
Lead acetate ($10^{-3}M$)	0
Malonate ($10^{-2}M$)	100

globin synthesis were reported (*8*). Whether the observed inhibition was due to the direct effect upon the synthesis of protoporphyrin or to the indirect action is to be decided by further experiments.

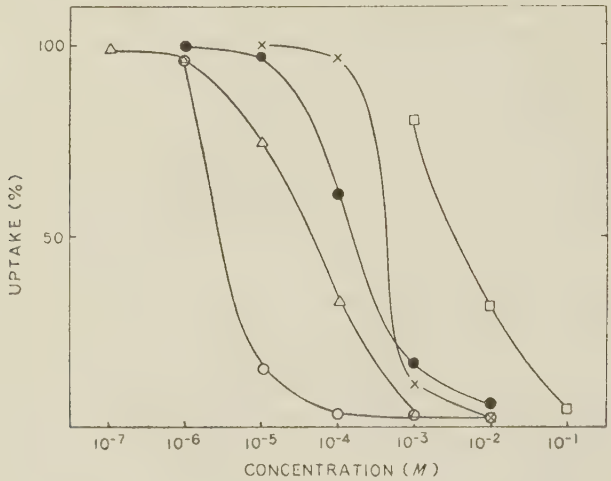


FIG. 2. Influence of inhibitors on the iron incorporation into heme in canine reticulocytes. —○— lead acetate, —△— 2,4-dinitrophenol, —□— fluoride, —×— cyanide, —●— fluoroacetate.

SUMMARY

1. Hemoglobin synthesis was studied *in vitro* in canine reticulocytes with the aid of radioactive iron. Time course experiments showed the iron uptake into hemin and non-hemin iron of the cells.

2. Among the inhibitors examined, malonate ($10^{-2}M$) and thyroxine ($10^{-5}M$) were without effect. *o*-Phenanthroline ($10^{-3}M$), diethyldithiocarbamate ($10^{-2}M$) and atebrin ($10^{-2}M$) completely inhibited the incorporation. The concentration for 50 per cent inhibition in cases of lead, 2,4-dinitrophenol, fluoroacetate, cyanide and fluoride were $10^{-5.5}M$, $10^{-4.5}M$, $10^{-4}M$, $10^{-3.5}M$ and $10^{-2.5}M$ respectively.

The authors express their thanks to Prof. S. Okinaka and Prof. H. Yoshikawa for their interests during this work. Thanks are also due to Drs. M. Hattori, Y. Kagawa and S. Morimoto for their cooperations.

REFERENCES

(*1*) Borsook, H., Deasy, C.L., Haagen-Smit, A.J., Keighley, G., and Lowy, P.H., *J. Biol. Chem.*, **196**, 669 (1952)

(*2*) Koritz, S.B., and Chantrenne, H., *Biochim. et Biophys. Acta*, **13**, 219 (1954)

(*3*) Walsh, E.D., Thomas, S.K.C., Fluharty, R.G., and Finch, C.A., *Science*, **110**, 396 (1949)

(*4*) Neuberger, A., in "Isotopes in Biochemistry" J. & A. Churchill, London,

68 (1951)
(*5*) Tanaka, S., and Yoneyama, Y., *J. Japan. Biochem. Soc.* (*in Japanese*) **29**, 954 (1958)
(*6*) Nakao, K., *Saishin-Igaku*, (*in Japan*) **11**, 129 (1956)
(*7*) Jensen, W.N., Ashenbrucker, H., Cartwright, G.E., and Wintrobe, M.M., *J. Lab. Clin. Med.*, **42**, 833 (1953)
(*8*) Nizet, A., *Bull. soc. chim. biol.*, **39**, 265 (1957)
(*9*) Yoshiba, A., Hattori, M., Nakao, K., Morimoto, S., Kagawa, Y., Minakami, S., and Yoneyama, Y., *J. Japan. Biochem. Soc.* (*in Japanese*) **30**, 297 (1958)

The Journal of Biochemistry, Vol. 45, No. 11, 1958

PAPER CHROMATOGRAPHIC SEPARATION OF ACID-SOLUBLE PHOSPHORUS COMPOUNDS*

By TOYOZO SEKIGUCHI, KANJI MIYAMOTO, MAKOTO NAKAO and HARUHISA YOSHIKAWA

(From the Department of Physiological Chemistry and Nutrition, Faculty of Medicine, University of Tokyo, Tokyo)

(Received for publication, April 14, 1958)

Radioactive phosphorus has been employed in a number of studies to obtain new informations about phosphorus metabolism in various organisms. In majority of the studies hitherto are applied fractionation procedures based on difference in solubility of phosphorus compounds and differential hydrolysis of organic phosphates (*1—3*). The classical methods, however, do not permit satisfactory separation of the phosphorus compounds containing radioactivity, owing to co-precipitation and incomplete precipitation of the salts of organic phosphates. Recently, separation of various phosphorus compounds have been improved by paper chromatography (*4*, *5*), ion exchange resin chromatography (*6*, *7*), and paper electrophoresis (*8*, *9*). Sekiguchi (*10*) and Mano (*11*) have developed a paper chromatographic method for the separation of acid-soluble phosphorus compounds such as inorganic phosphates, sugar phosphates and nucleotides in trichloracetic acid extracts of animal tissues and yeast cells. This method has been routinely employed in tracer experiments with radio-isotopes in the authors' laboratory over four years with satisfaction.

The present paper is concerned with paper chromatographic procedures for the separation of the biologically important acid-soluble phosphorus compounds.

EXPERIMENTAL

Procedure

Filter Paper—Toyo Filper Paper No. 3 which has been washed previously as follows is used. Five 60×60 cm. sheets are hung, washed down with 500 to 1000 ml. of 0.1 *N* HCl in the same way as in descending chromatography, then with redistilled water until the washings no longer contain chloride ion and dried in an airy place. The wash with hydrochloric acid removes phosphorus containing substance from the paper and reduces the ultravioloet light absorption background of the chromatogram, in addition improving the separation of spots.

Chromatography—A sample is put on an appropriately trimmed sheet of the paper in a line 2 to 4 cm. in length and 6 cm. above the shorter edge by means of a capillary,

* This work was supported by a grant from the Ministry of Education.

and dried in a current of air. It is advisable that the volume of the sample does not exceed 0.3 ml. If the material is provided as an insoluble salt such as barium or lead salt it must be previously decationized by treating the suspension of the salt with Amberlite IR 120 (H^+ form).

The following solvents are used for developing the paper chromatograms.

A. *iso*-Propanol-*iso*-Amyl alcohol-trichloracetic acid (5 per cent, *w/v*)-lactic acid (72 per cent, *w/w*), 15 : 5 : 10 : 0.5, according to Sekiguchi and Mano (*10*, *11*). Ammonia has been omitted from the original solvent system, since it does not make and difference in the results. The chromatogram is allowed to develop in a glass cylinder at 0–5° in a refrigerator until the solvent front reaches 37 cm. above the original line, the time being about 72 hours.

B. *iso*-Butyric acid-0.5 *N* ammonia, 20 : 12 (pH, 3.6), according to Magasanik *et al.* (*12*). This solvent which has introduced originally for descending chromatography is applied to ascending technique in this study, the chromatogram being run at 15° for 20 hours.

C. *n*-Butyric acid-0.5 *N* ammonia, 20 : 12 (pH, 3.6). The substitution of *n*-butyric acid for more expensive isobutyric acid in the solvent B has improved the reproducibility of the chromatogram (*13*).

D. *n*-Propanol-concentrated ammonia-water, 20 : 10 : 3, according to Hanes and Isherwood (*14*). Developing time is 20 hours at room temperature.

E. Tertiary butanol-picric acid-water, 20 ml. : 1 g. : 15 ml., according to Hanes and Isherwood (*14*). The chromatogram is run at room temperature for about 30 hours. This solvent is suited for the separation of sugar phosphates.

One frequently encounters substances which are of similar R_f value. If these are of low value, then it may be possible to separate them by means of "double passes" (*11*, *15*).

The paper taken out of the glass cylinder is prefearbly rinsed by passing through purified ether in a tray rapidly and evenly and then dried in a current of air. The ether treatment is advantageous to remove the solvent which may interfere with further examination such as location of a spot by ultraviolet light absorption, and to cut down the time necessary for drying the chromatogram.

Location of Spots—The procedures which are used to locate the spots are (a) spraying with appropriate reagents (b) use of ultraviolet light and (c) radioautography. If the spots are to be eluted from the chromatogram for further studies spraying with coloring reagents should be avoided.

(*a*) *Spray*: Phosphorus compounds are made visible by Bandurski and Axelrod's methods (*16*): The thoroughly dried sheet is sprayed with Hanes and Isherwood's perchloric acid-molybdate reagent (*14*), heated at 80° and then exposed to a germicidal lamp. This enables detection of phosphorus down to 5 μg., resulting in the appearance of blue colors.

Not less than 20 μg. of phosphorylated sugars can be detected by spraying with *p*-anisidine-HCl (5 per cent in butanol) solution and then heating at 80°. The colors are yellow for fructose, brown for glucose and red for pentose.

(*b*) *Ultraviolet Light*: The detection of nucleotides nucleosides and free bases is achieved by ultraviolet light, by noting the dark spots on a fluorescing background of the paper. Light emitted from a germicidal mercury are lamp is passed through a Markham and Smith's liquid filter (*17*), of which chlorine gas chamber is omitted as described by Makino (*18*). For a permanent record a contact print upon X ray film is made (*19*). As little as 5 μg. of purines can be detected easily by this technique.

Some compounds show characteristic fluorescence under ultraviolet light: riboflavin

derivatives—green; guanine derivatives—blue-violet.

(*c*) *Radioautography*: When radioactive isotopes are employed the chromatogram is radioautographed by contact technique with Fuji No-Screen X-Ray Film No. 400. This enables the location of a minute quantity of the compound containing radioactivity. For the P^{32} studies, original radio-activity of about 3,000 c.p.m. at a distance of 5 cm. from a thin window Geiger-Muller counter is sufficient to obtain an adequate radioautogram with exposure of several hours.

Elution for Rechromatography—The elution of a spot from the chromatogram is conveniently effected by capillary elution with 0.1 *N* HCl at low temperature. Two tenths ml. of HCl is sufficient to accomplish the elution, the eluate being ready for rechromatography.

Application to Trichloracetic Acid (TCA) Extract—To the ice-cold TCA extract from biological material is added an excess of 25 per cent barium acetate solution, the mixture is neutralized to pH 8.2 by addition of KOH with phenolphthalein as an indicator and after addition of 4 volumes of ethanol placed in a refrigerator overnight to complete the precipitation of barium salt which corresponds to "barium-insoluble fraction" plus "barium-soluble and alcohol insoluble fraction" of Schneider and LePage (*3*).

The precipitate is washed with a small volume of water and dissolved in a minimum quantity of 0.1 *N* HCl. If the washing with water is omitted much more HCl is needed to dissolve the salt because of the presence of ethanol. It is desirable, therefore, to dissolve the precipitate with constant stirring in an ice bath. The solution is freed from barium ion by addition of 0.1 *N* sulfuric acid and centrifugation, and then treated with Amberlite IR 120 (H^+ form) by batch technique in order to remove the last trace of cations. When the final volume exceeds 2.5 ml. it is preferable to concentrate the solution by lyophilizing and dissolve the residue in a small amount of water. A known quantity of the fluid is subjected to paper chromatography as described above.

RESULTS AND DISCUSSION

The R_f values of the known phosphorus compounds in various solvent systems are presented in Table I, and those of purines, pyrimidines and nucleosides in Table II. The values in these tables are by no means definite but may vary under different conditions.

The solvent system (A), *iso*-propanol-*iso*-amyl alcohol-trichloracetic acid-lactic acid, of Sekiguchi and Mano is a modification of Ebel's acid solvent consisting of 74 ml. of *iso*-propanol, 5 g. of trichloracetic acid, 0.3 ml. of conc. ammonia and 25 ml. of water (*22*). The addition of lactic acid to the original Ebel's solvent mixture improves the separation of phosphorus compounds but results in too higher R_f values. This disadvantage has been overcome by replacing a portion of *iso*-propanol with *iso*-amyl alcohol. In this solvent the R_f values of nucleotides and sugar phosphate are rather evenly distributed from 0 to 0.8 and a better chromatogram than in other solvents is obtained when this is used as a first developing solvent for a study of phosphorylated compounds in biological materials.

Hanes and Isherwood's *n*-propanol-ammonia-water system (D) results in also a good separation, but has disadvantages that the R_f values are lowar and the higher alkalinity of the solvent may cause partial hydrolysis of labile phosphates during the chromatographic procedure.

TABLE I

R_f Values of Phosphorus Compounds in Different Solvent Systems

Compounds	A	B	C	D	E
Inorganic phosphates					
Orthophosphate	0.65	0.31	0.42	0.12	0.71
Pyrophosphate	0.43	0.25	0.34		
Nucleotides					
Adenosine-5′-monophosphate	0.37	0.60	0.62	0.22	
Adenosine-3′-monophosphate	0.39	0.62	0.62		
Adenosine diphosphate	0.25	0.49	0.53		
Adenosine triphosphate[1]	0.09	0.40	0.48		
Inosine-5′-monophosphate	0.30	0.40	0.42	0.41	
Guanosine-3′-monophosphate	0.25			0.12	
Uridine-3′-monophosphate	0.49	0.38	0.40	0.40	
Uridine diphosphate	0.32	0.20			
Cytidine-3′-monophosphate	0.42				
Flavin mononucleotide	0.28				
Flavin adenine dinucleotide	0.02				
Diphosphopyridinenucleotide	0.03				
Triphosphopyridinenucleotide	0.02				
Thiamine pyrophosphate	0.24				
Sugar phosphates and related compounds					
Glucose-1-phosphate	0.31		0.34	0.24	0.39
Glucose-6-phosphate	0.40	0.25	0.31	0.21	0.38
6-Phosphogluconate[2]	0.44				0.37
Fructose-6-phosphate	0.44	0.22	0.35	0.27	0.48
Fructose-1, 6-diphosphate	0.55	0.25	0.22	0.10	0.51
Ribose-5-phosphate	0.47	0.35	0.38	0.27	0.44
3-Phosphoglycerate	0.59		0.38	0.21	0.58
2, 3-Diphosphoglycerate	0.44	0.19		0.05	0.60
Phosphoenolpyruvate	0.78				
Propandiolphosphate	0.68				0.84
Creatine phosphate	0.43				

A, *iso*-Propanol-*iso*-amyl alcohol-trichloracetic acid-lactic acid.
B, *iso*-Butyric acid-0.5 *N* ammonia.
C, *n*-Butyric acid-0.5 *N* ammonia.
D, *n*-Propanol-ammonia-water.
E, *tert*-Butanol-picric acid-water.
1) Prepared from rabbit and dog muscles (*20*).
2) Prepared from glucose-6-phosphate by bromine oxydation (*21*). Flavin mono-

nucleotide was kindly provided by Dr. T. Sato of Tokyo College of Technology; fiavin adenine dinucleotide by Dr. K. Yagi of the Faculty of Medicine, University of Nagoya; glucose-1-phosphate by Dr. N. Hosoya and Dr. G. Urata of the Department of Biochemistry, University of Tokyo: 2, 3-diphospho-glycerate by Dr. S. Akamatsu of the Department of Biochemistry, University of Chiba; phosphoenolpyruvate by Dr. Y. Mano of the Department of Biochemistry, University of Tokyo. Other samples were provided commercially.

TABLE II

R_f Values of Purines, Pyrimidines and Nucleosides in Different Solvent Systems

Compounds	A	B	D
Adenine	0.72		0.54
Guanine	0.46		
Hypoxanthine	0.47		
Uric acid	0.30		
Thymine	0.61	0.62	
Adenosine	0.50	0.72	0.72
Guanosine	0.45	0.72	
Uridine	0.55	0.68	
Cytidine	0.50	0.77	
Thymidine	0.60		

See Table I for the definition of the solvent systems. The samples are provided commercially.

Even the best chromatogram, however, enables hardly a complete separation of closely running compounds in biological tissues. One needs, therefore, to resort to elution and rechromatography of the spots, employing various developing solvents which are suited for either nucleotides or sugar phosphates. For the purpose of separating nucleotides. Magasanik's solvent (B) was used at first. Later *n*-butyric acid was used instead of *iso*-bytyric acid according to Ohmura and Fukushi (*13*) in the solvent because the modified procedure gave more reproducible results which made up for the tendency to gathering of spots to the middle part of the chromatogram.

Sugar phosphates are separated by rechromatography in either Hanes and Isherwood's *n*-propanol-ammonia-water system (D) or *tert*-butanolpicric acid-water system (E). The latter is superior to the former in that spots with higher R_f values are obtained with good separation but the presence of picric acid interferes iometimes the further examination of the compounds.

When elution and rechromatography are performed care must be taken to minimize the decomposition of easily hydrolysable phosphorus compounds such as adenosine triphosphate, adenosine diphosphate and creatine phosphate,

Many of such unstable compounds are of biological importance and thein decomposition products may play rôles entirely different from the mother substances. This is the main difficulty encountered in paper chromatographic techniques for the investigation of phosphorus compounds in biological materials, in contrast to more stable ones like amino acids, carbohydrates *etc.*

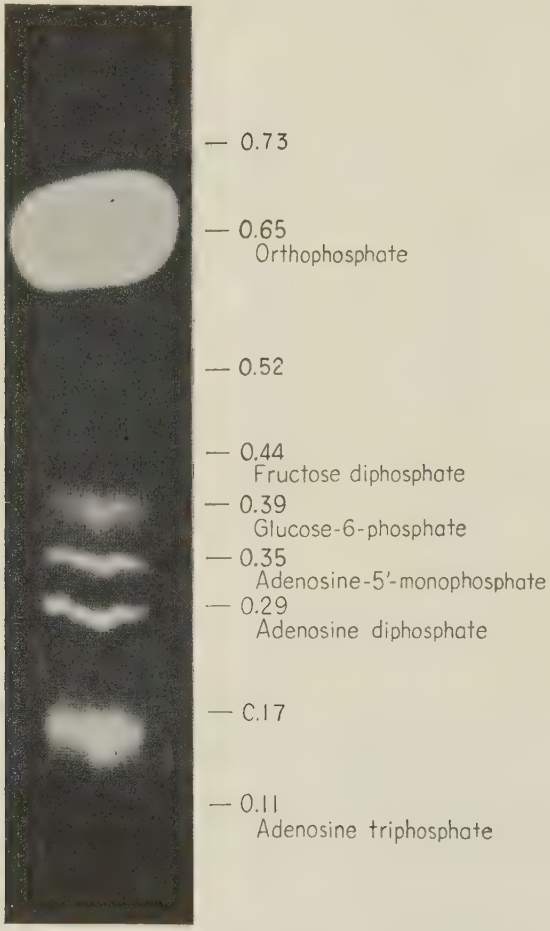


Fig. 1. Radioautochromatogram of acid-soluble phosphorus compounds in boar spermatozoa incubation with P^{32}.

Keeping the temperature as low as possible during the procedures and washing the developed paper with pure ether before drying are effective to prevent decomposition of unstable phosphates.

In Fig. 1 is illustrated an example of radioautogram of paper chromatogram run in the solvent A obtained from the trichloracetic acid extract of boar spermatozoa which were incubated with radioactive phosphate. Nine radio-

active spots were obtained of which five were tentatively regarded as adenosine triphosphate, adenosine diphosphate, glucose-6-phosphate, fructose-1, 6-diphosphate and inorganic orthophosphate. It was elucidated by elution and rechromatography with solvent B that the radioactive spot corresponding to strongly radioactive glucose-6-phosphate overlapped with the ultraviolet absorbing, weakly radioactive spot of adenosine-5′-monophosphate. The compound with R_f value of 0.17 gave an ultraviolet absorption curve corresponding to an adenine nucleotide, but its real nature is still undissolved.

SUMMARY

Paper-chromatographic procedures for the separation of acid-soluble phosphorus compounds in biological tissues are described. *Iso*-propanol-*iso*-amyl alcohol-trichloracetic acid-lactic acid system was proved to be suitable for the first development of the chromatogram.

In addition, R_f values of biologically important phosphorus compounds in five different solvent systems are presented. An example of radioautogram of paper chromatogram obtained from the trichloracetic acid extract of boar spermatozoa was also illustrated.

REFERENCES

(*1*) Eggleton, G.P., and Eggleton, P., *J. Physiol.*, **63**, 193 (1929)
(*2*) Kaplan, N.O., and Greenberg, D.M., *J. Biol. Chem.*, **156**, 511 (1944)
(*3*) Schneider, W.O., and Le Page, G.A., in Manometric Techniques and Related Methods for the Study of Tissue Metabolism, Ed. by Umbreit, W.W., Burris, D.U., and Stauffer, Burgess Publ. Co., Minneapolis, 2nd ed p. 185 (1950)
(*4*) Cohn, W.E., and Carter, C.E., *J. Am. Chem. Soc.*, **72**, 4273 (1950)
(*5*) Eggleston, L.V., and Hems, R., *Biochem. J.*, **52**, 1 (1952)
(*6*) Cohn, W.E., *J. Am. Chem. Soc.*, **72**, 1471 (1950)
(*7*) Hurlbert, R.B., Schmitz, H., Brumm, A., and Potter, V.R., *J. Biol. Chem.*, **209**, 23 (1954)
(*8*) Markham, R., and Smith, J.D., *Biochem. J.*, **52**, 552 (1952)
(*9*) Berg, P., and Joklik, W.K., *Nature*, **172**, 1008 (1953)
(*10*) Sekiguchi, T., *J. Japan. Biochem. Soc.* (*in Japanese*), **26**, 476 (1954)
(*11*) Mano, Y., *J. Japan. Biochem. Soc.* (*in Japanese*), **27**, 62 (1955)
(*12*) Magasanik, B., Vischer, E., Doriyu, R., and Chargaff, E., *J. Biol. Chem.*, **186**, 37 (1950)
(*13*) Ohmura, K., and Fukusai S., *J. Agr. Chem. Soc. Japan* (*in Japanese*), **27**, 515 (1953)
(*14*) Hanes, O.S., and Isherwood, F.A., *Nature*, **164**, 1107 (1949)
(*15*) Geans, A., Wise, S., and Dimler, S., *J. Anal. Chem.*, **23**, 415 (1951)
(*16*) Bandurski, R.S., and Axelrod, R., *J. Biol. Chem.*, **193**, 405 (1951)
(*17*) Markham, R., and Smith, J.D., *Biochem. J.*, **45**, 294 (1949)
(*18*) Makino, K., *J. Japan. Biochem. Soc.* (*in Japanese*), **27**, 489 (1955)
(*19*) Markham, R., and Smith, J.D., *Biochem. J.*, **49**, 407 (1951)
(*20*) Seegmiller, J.E., and Horecker, B.L., *J. Biol. Chem.*, **192**, 175 (1951)
(*21*) Carter, C.E., Biochemical Preparations, John Wiley & Sons. Inc., New York Vol. **I**, 5 (1949)
(*22*) Ebel, J.P., *Bull. soc. chim. biol.*, **34**, 321 (1952)

The Journal of Biochemistry, Vol. 45, No. 11, 1958

ON THE NUCLEIC ACIDS OF MICROBIAL ORIGIN SEPARATED BY USE OF CONTINUOUS PAPER ELECTROPHORESIS

By SHUZO AKASHI and HANAKO YOKOI

(From the Department of Biochemistry, School of Medicine, Nagoya City University, Nagoya)

(Received for publication, April 7, 1958)

The protein-chloroform gel method is the most excellent and common technique introduced by Sevag (*1*) to remove proteins from nucleoproteins. However, it is not an easy task even by this means to eliminate trace amounts of proteins from nucleic acid preparations. In addition there may be some cases where this technique fails to be fully effective. In this connection we have attempted to utilize the technique of continuous paper electrophoresis for the isolation of nucleic acids from nucleoproteins or nucleic acids contaminated with proteins, all of which had been derived from various kinds of microorganisms. As a result of this it has been found that this method affords an useful means for isolating nucleic acids free of proteins in a satisfactory yield and further that all the nucleic acids studied liberated on acid hydrolysis in a yield of some 1.5 per cent amino acids of several kinds resulting presumably from hydrolysis of polypeptides chemically bound to the original nucleic acids.

EXPERIMENTAL AND RESULT

Preparation of Bacterial Material—Bacteria chosen for the present research were *a*) *Staphylococcus aureus*, *b*) *B. pullorum*, *c*) *Alcaligenes faecalis*, *d*) *Mycobacterium tuberculosis hominis*, *e*) *Mb. tuberculosis avium*, and *f*) *Mb. phlei*. Bacterical cells were grown on an ordinary agar plate (*a* and *b*), in an ordinary bouillon (*c*) or using the Long's synthetic medium (*d*, *e*, and *f*) to obtain maximum growth. The grown organisms were collected by centrifugation and washed several times with 0.1 *N* acetic acid. The moist bacterial mass was lyophilized and the dried material was ground using a stainless steel ball mill until a gray white powder of disintegrated bacterial cells was obtained. Finally it was defatted by treatment with ten times the sample weight of ethanol-ether (1 : 1) several times at room temperature.

Isolation of Nucleoproteins—The defatted bacterial powder was shaken vigorously with ten times its weight of 0.1 *N* acetic acid for several hours and centrifuged. The separated extraction residue was subjected to a similar extraction procedure with twenty times its weight of 1/15 *M* phosphate

buffer (pH 6.6). The slightly turbid supernatant which separated out upon centrifugation of the bacterial suspension in phosphate was further centrifuged at 14.000 r.p.m for 30 minutes to provide an entirely clear fluid. The latter solution was made acid (pH 2.4–2.6) with glacial acetic acid, yielding a flocculent precipitation of nucleoproteins which was separated, washed with 0.1 *N* acetic acid and dehydrated with ethanol and ether to make a white powder. The yields as well as the contents of N and P of the respective nucleoprotein samples isolated from various bacteria above mentioned are given in Table I.

TABLE I

Nucleoproteins Isolated from Several Bacteria

Species of bacteria	Yield in per cent on a dry weight, basis of bacteria	N content* per cent	P content** per cent
Staphylococeus aureus	8.8	15.2	2.97
B. pullorum	3.6	14.1	3.05
Alcaligenes faecalis	11.1	14.4	3.90
Mb. tuberculosis hominis	3.4	13.5	2.83
Mb. tuberculosis avium	3.0	13.7	2.56
Mb. phlei	4.1	15.5	3.10

* Measured by an improved Kjeldahl-Nessler method (*3*).
** Measured by the Fiske and Subbarow method (*4*).

Isolation of Crude Nucleic Acids as Copper Salt from the Filtrate of Nucleoprotein Precipitate—It is usual that free nucleic acids occur in the filtrate of nucleoprotein precipitate. Therefore this fraction may be referred to as the free nucleic acid fraction. To obtain this nucleic acid in a crude form 20 per cent copper chloride was added to the filtrate from the nucleoproteins obtained above, the precipitated copper nucleate was separated by centrifugation, washed with water, suspended in a small amount of phosphate buffer (pH 7.7) and decomposed by the addition of 20 per cent sodium sulfide, care being taken so that the pH of the medium does not exceed 8.0. After removal of copper sulfide by centrifugation the supernatant was subjected to continuous paper electrophoresis to obtain a protein-free sample of nucleic acid according to the technique to be described in the following which permits the separation of nucleic acid from the nucleoproteins.

Tentative Experiment on the Fractionation of Microbial Nucleoproteins by Paper Electrophoresis—This is the fundamental procedure to be carried out prior to the fractionation studies of nucleoproteins by continuous paper electrophoresis and this will permit the necessary information as to the practicing of the latter method. It was made clear that the nucleoproteins from bacterial sources are separable into two major components under the experimental conditions given below.

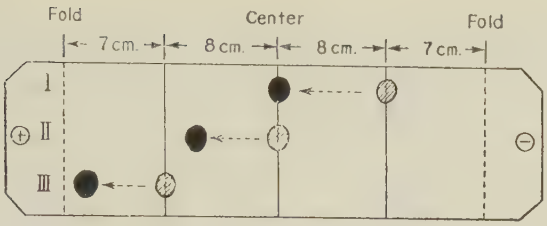


FIG. 1. Paper electrophoretic patterns of nucleoproteins from *Staphylococcus*. Dark spots: ultraviolet light absorbing areas (nucleic acids) moved towards the cathodic side. Hatched spots: ninhydrin positive areas (proteins) remained immobile, at the starting points. Arrows shows the direction of migration. Experimental condition: phosphate buffer of pH 7.7, and I=0.19; field strength 10 volts/cm. Sample application: 0.01 ml. of 15 per cent solution of nucleoproteins in phosphate buffer of pH 7.7, corresponding to approximately 150 μg. of sample, was applied at three different points; I (anodic side), II (center), and III (cathodic side).

Operation of Continuous Paper Electrophoresis—The apparatus employed in the present research was manufactured in our laboratory mainly following the design given by Holdsworth (*2*). The simplify the apparatus thus reducing the expense of construction several modifications were made in many respects; the material of framework, cooling device, electrode construction, sample feeding machine *etc.* Mention will be made here in some detail about the analytical procedures involved in employing this apparatus.

(*a*) *Conditioning of the Apparatus Prior to the Test*—It is only natural that the apparatus should be placed in a vertical position. A sheet of Toyo Filter Paper No. 5A of thin coarse textile, 42×44 cm., was serrated along one of its shorter edges to afford 29 drip points. To fix the paper in a right position on the vertical surface of the cooler, at first the unserrated upper edge was dipped into the electrolyte through which was located on the top of the cooler carrying the phosphate buffer of pH 7.7 so that the serrated lower edge of the paper might just come down the bottom edge of the cooler. Thus the paper was hung just like a curtain very closely to the cooler surface. The phosphate buffer, the same as that in the trough, was sprayed heavily and diffusedly all over the paper, so that the entirely wet paper might be adhered to the cooler surface. Then the paper was pressed against the surface with a rubber roller commonly in use for photography to eliminate all the air pockets produced between the paper and the cooler surface as well as the excess electrolyte absorbed within the paper. The 29 drip points were then pushed into the corresponding collecting funnels standing just below so that all the points might touch the collector walls. Then two electrode tubes carrying platinum wire were fixed tightly with cramps to the paper curtain, each one along either side edge of the paper. Finally the front glass window of the apparatus was closed to make the

apparatus a closed system and the current switched on. When a steady state had been established in the apparatus, for which usually several hours were required, sample feeding was started.

(*b*) *Preparation of Sample Solutions*—The concentration of nucleoprotein solutions suitable for a complete separation of nucleic acids from proteins ranged from 20 to 40 mg. per ml. Since usually for sample application a 5 ml. supply syringe was employed, approximately 100–200 mg. of nucleoproteins were able to be treated in one experiment. These amounts were corresponding to 10–40 mg. of nucleic acids, as the nucleoproteins under study contained 10–20 per cent nucleic acids. Therefore a sample solution was prepared by dissolving approximately 150 mg. of nucleoproteins to be analyzed in 6 ml. of phosphate buffer of pH 7.7 which contained 0.02 *M* concentration of sodium citrate to inhibit nuclease activity. In case nucleic acid especially yeast RNA rather than nucleoproteins was to be used as a sample for removing slight impurities, considerably higher concentrations, *i.e.*, 20–50 mg. of nucleic acid per ml. were found applicable, as illustrated in Fig. 2.

(*c*) *Sample Feeding*—This was conducted by means of a syringe mechanically operated at a constant rate. For quantitative purpose a known volume, *e.g.*, 4.0 ml. of sample solution was withdrawn into the supply syringe through the transfer capillary tube connected with the syringe by a rubber tubing. The syringe was mounted on the syringe holder. The transfer tube had a rubber stopper and the glass plate of the front window was provided with several bores for inserting the rubber stopper. Next the transfer tube was fixed to the glass plate by setting the rubber stopper to the selected bore and the tube was pushed in so far as its tip came just in contact with the paper curtain. Then the plunger of the syringe was set in motion by spring works. Upon evacuation of the syringe, for which some 15 hours were required in the present experiments, the syringe was washed three times with about 0.3 ml. portions of the phosphate buffer.

(*d*) *Subsequent Prolonged Electrophoresis*—After the washing procedure was completed, the electrophoresis was still to be continued further at least 9 hours before it came to an end. This additional prolonged run of electrophoresis was found necessary to obtain a higher yield of sample, as evidenced by the recovery test to be mentioned below.

(*e*) *Detection of the Drip Points by the Reprica Method*—Prior to the washing procedure it was necessary to provide information as to the drip points where the separated individual substances were likely to assemble. For this purpose the replica method seemed convenient, although it was available for qualitative rather than for quantitative works. A strip of filter paper about 20×7 cm. with a serrated edge similar to that of the paper curtain was made wet with the phosphate buffer and placed on the curtain so that each notch of both serrated papers might fall on one another and adhere closely together. Upon continuing of electrophoresis for 10 to 15 minutes, the duplicate sheet of paper was removed from the paper curtain, dried

over an electric heater and examined under ultraviolet light through a 253.7 mμ short-wave filter. It was usual to locate nucleic acids as an appreciably demarcated dark zone ranging from 3 to 4 drip points in the vicinity of drip points No. 9 to No. 11, as shown in Fig. 2.

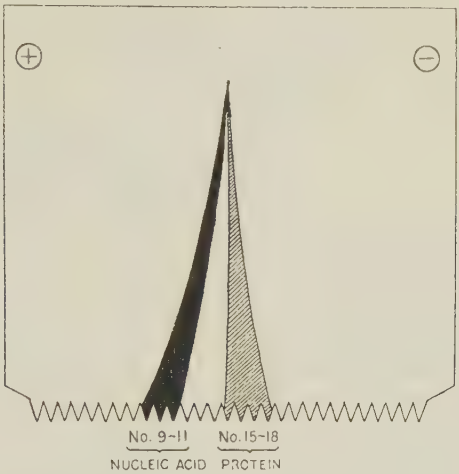


Fig. 2. Resolution of staphylococcus nucleoproteins into nucleic acid and protein moieties by continuous paper electrophoresis. The dark zone extending between drip points No. 9–11 is the track run by nucleic acids, inspected under ultraviolet light through a 253.7 mμ short wave filter. The hatched zone extending between drip points No. 15–18 represents the passage of proteins visualized by the ninhydrin reaction. Buffer solution, phosphate buffer of pH 7.7 and ionic strength 0.19; Paper, Toyo Paper No. 5A, 42×44cm.; Applied voltage, 400 volts; Current, 60 mA; Rate of sample feeding, 1 ml./3 hours; Sample applied, 4 ml. of 3 per cent solution of staphylococcus nucleoproteins in phosphate buffer (pH 7.7) which is 0.02 *M* with respect to sodium citrate.

Recovery Test for the Separation of Yeast RNA by Continuous Paper Electrophoresis—Sodium salt of RNA from yeast (E. Merck) of P content 7.43 per cent and N content 14.83 per cent was once subjected to purification procedures by precipitation of the free acid and conversion into its sodium salt, through which a preparation of 8.46 per cent P and 14.37 per cent N was obtained.

This purified sample of sodium ribonucleate was dissolved in 1/15 *M* phosphate buffer of pH 7.7 to give a solution containing 30 mg. of sample per ml. 4.0 ml. of this solution corresponding to 120 mg. of material were taken into the syringe and applied to continuous paper electrophoresis at a rate of 1 ml. per 3 hours, following the manner mentioned above. Upon completion of the washing procedure of the ryringe, densitometric measurement with the combined sample solution was carried out in high dilutions

and the subsequent electrophoresis was continued with densitometric checking at a 3 hour interval until there was no increase in the optical density of the whole combined sample solution. The final value was compared with that of the original specimen applied, the results of which are indicated in Table II.

Table II

Recovery Test for the Separation of Yeast RNA by Continuous Paper Electrophoresis

Types of solution	Extinctions ($\times 10^{-3}$) of the combined appropriate effluents collected from the beginning at 3 hour intervals				
Time elapsed after the end of washing in hour	0	3	6	9	12
I RNA solution applied	0.520	—	—	—	—
II Combined effluents from drip points No. 9–11 with application of RNA	—	0.552	0.628	0.658	0.657
III Combined effluents from drip points No. 9–11 without application of RNA (blanks)	—	0.140	0.182	0.184	0.184
Actual recovery, II—III (per cent, II—III/I$\times$100)		0.412 (79.3)	0.446 (85.8)	0.470 (90.4)	0.478 (91.0)

This table indicates that yeast RNA can be recovered in a yield of about 90 per cent under the experimental conditions mentioned above, provided the subsequent prolonged electrophoresis was run for 9 hours. Accordingly, when dealing with RNA in amounts of about 120 mg. at a time, at least approximately 27 hours are required, of which 3 hours for conditioning of the apparatus, 12 hours for sample feeding, 3 hours for syringe washing (0.3 ml.$\times$3) and 9 hours for subsequent paper electrophoresis. As shown in the above figure the separated proteins appeared in the region of No. 15–18, in which the paper gave a positive coloration for the ninhydrin reaction.

Preparation of Sodium Nucleate from Effluents—The effluents from the ultraviolet absorbing drip points No. 9–11 were combined, the total volume amounting to some 25 to 30 ml. and the whole solution was dialyzed against distilled water in the refrigerator with frequent renewal of outside water until there was no trace of phosphate ion in the dialysates, for which several days were needed. The non-dialyzable fraction of the effluents was vacuum concentrated in a desiccator over NaOH and P_2O_5 to a syrupy fluid, to which ethanol was added to form a white fibrous precipitate of sodium nucleate. The latter substance was dehydrated by treatment with ethanol and ether and analyzed for N and P contents. Table III illustrates the analytical results of the nucleic acids isolated from several microorganisms being studied.

TABLE III

Analyses for N and P on the Nucleic Acids Isolated from Several Bacteria by Continuous Paper Electrophoresis

Species of bacteria	Yield in per cent on a dry weight basis of nucleoprotein	N content (per cent)	P content (per cent)	Atomic N : P ratio
Staphylococcus aureus	20.0	13.09	7.70	3.74
B. pullorum	14.7	12.82	8.08	3.48
Alcaligenes faecalis	28.5	13.53	7.10	4.29
Mb. tuberculosis hominis	11.8	14.47	8.68	3.67
Mb. tuberculosis avium	10.7	15.40	8.43	4.03
Mb. phlei	15.3	13.50	7.43	4.00

Negative Test for Proteins by the Sevag's Method—A solution of 30 mg. nucleic acid sample per ml. of phosphate buffer solution of pH 6.0 was vigorously shaken with one half its volume of chloroform-octanol mixture (8 : 1) without forming any protein-chloroform gel. Control experiments showed that a solution containing nucleoproteins in amounts more than 20 μg. per ml. gave a positive reaction for protein by the Sevag's method. Considering the protein content in this nucleoprotein sample, the detection limit for proteins by this sensitive protein reaction may be around 16 μg. protein per ml.

Amino Acid Contents in Various Microbial Nucleic Acids Obtained from the Nucleoprotein as well as the Free Nucleic Acid Fraction—About 3 mg. of sample were hydrolyzed with 0.3 ml. of 6 *N* HCl in a sealed tube at 100° for 20 hours. The hydrolysate was vacuum evaporated to dryness in a desiccator over NaOH and P_2O_5 several times repeatedly with the addition of water to remove the excess hydrochloric acid. The final evaporation residue was dissolved in a small amount of water, the pH was adjusted to 5.0 with NaOH and the total volume was made up to 2 ml. with water to provide a test solution for quantitative estimation of amino acids.

1 ml. of the test solution containing about 1.5 mg. of sample was evaporated to dryness with the addition of 0.06 ml. of 1 *N* NaOH on a boiling water bath to remove ammonia present in it and the residue was taken up in 1 ml. of water. There were added 1 ml. of 1 per cent ninhydrin in ethylene glycol—pH 5.0 citrate buffer (1 : 1) and 0.05 ml. of 200 mg. stannic chloride in 12.4 ml. citrate buffer and the mixture was heated at 100° for 30 minutes. Upon cooling, distilled water was added to make the volume up to 3.5 ml. and the optical density was measured by a Beckmann type spectrophotometer at 570 mμ. As a standard 0.1 μM solution of L-leucine was employed. The results are summarized in Table IV.

Qualitative Estimation of the Total Amino Acids Present in the Microbial Nucleic Acids—Analyses were made with the staphylococcus nucleic acids as well as the yeast RNA. The concentrate of the hydrolysate of 50 mg. sample obtained

TABLE IV

Amino Acid Contents in the Hydrolysates of Various Microbial Nucleic Acids Obtained from the Nucleoprotein as well as the Free Nucleic Acid Fraction

Microbial source of nucleic acids	Amino acid content (%)
Staphylococcus aureus (*a*)	1.15
,, ,, (*a*)*	1.13
,, ,, (*b*)	1.20
B. pullorum (*a*)	1.85
,, ,, (*b*)	0.91
Alcaligenes faecalis (*a*)	0.80
Mb. tuberculosis hominis (*a*)	1.67
,, ,, ,, (*b*)	1.24
Mb. phlei (*a*)	1.42
,, ,, (*b*)	1.20
Yeast (*c*)	1.83
,, (*d*)	1.52

(*a*) The nucleic acid prepared from the nucleoprotein by a single performance of continuous paper electrophoresis.

(*a*)* The sample obtained by a double performance of the same procedure.

(*b*) The nucleic acid separated from the free nucleic acid fraction as a copper salt which was followed by decomposition with Na_2S and then by purification through continuous paper electrophoresis.

(*c*) Yeast RNA (E. Merck) deproteinized by the Sevag's method.

(*d*) Yeast RNA obtained by subjecting the specimen (*c*) further to continuous paper electrophoresis.

TABLE V

Amino Acids Found in the Hydrolysates of Staphylococcus Nucleic Acids and Yeast RNA

Amino acid fraction	Staphylococcus nucleic acids	Yeast RNA
Acidic	Aspartic acid, Glutamic acid	Aspartic acid, Glutamic acid
Neutral	Leucine and/or Isoleucine, Phenylalanine, Valine, Alanine, Glycine, Serine	Valine, Alanine, Glycine, Serine
Basic	Lysine	Histidine

in a similar manner just as mentioned above was taken up in 0.5 ml. of pyridine-acetic acid-water mixture pH 3.9 (3 : 10 : 500 *v/v*) and was subjected to continuous paper electrophoresis according to our modification of the Grassmann's method (*5*) to separate into three amino acid fractions,

i.e., an acidic, a neutral and a basic ones. Each fraction was analyzed for individual amino acids by one-dimentional paper chromatography. The data are as illustrated in Table V.

DISCUSSION

It has been demonstrate that the nucleoproteins as well as the nucleic acids contaminated with a small amount of proteins are able to be fractionated into nucleic acid and protein portions by continuous paper electrophoresis on a small preparative scale. However, the procedure is so tedious and time consuming that this method does not seem to be suitable for the purpose of a larger scale preparation.

A noticeable fact is that all the nucleic acid thus prepared from various microorganisms liberated upon acid hydrolysis amino acids which were estimated at approximately 1.5 per cent by the ninhydrin colorimetric method using leucine as a standard. Analysis of amino acids by paper chromatography was carried out with a hydrolyzed sample of staphylococcus nucleic acid leading to the identification of nine amino acids namely glycine, serine, alanine, valine, phenylalanine, leucine, aspartic acid, glutamic acid, and lysine.

The simplicity of the composition suggests that these amino acids might have originated from polypeptides rather than from proteins. It has been also shown that the nucleic acid sample obtained by a single performance of continuous paper electrophoresis contained as much amino acids as did the sample obtained by a double performance of the same procedure. On the other hand the staphylococcus nucleic acid obtained from the nucleoproteins by the S e v a g ' s procedure was further subjected to the electrophoretic procedure producing the sample of unchanged amino acid content.

Based on these results it is very likely that the nucleic acids being studied are in combination with some certain type of polypeptides which are inseparable in the electric field applied in the present research. It is also noticeable to find that the yeast nucleic acid investigated is accompanied by polypeptides in analogy to the nucleic acids of the other microorganisms before mentioned. The work on this interesting problem is now under way, the result of which will be published in the future paper.

SUMMARY

1. By means of continuous paper electrophoresis were separated the nucleic acids from the nucleoproteins or the nucleic acids slightly contaminated with proteins originating from various sources of microorganisms such as *Stapylococcus aureus*, *B. pullorum*, *Alcaligenes faecalis*, *Mb. tuberculosis hominis*, *Mb. tuberculosis avium*, and *Mb. phlei*.

2. All of the samples as well as the similarly treated yeast RNA have proved to contain some 1.5 per cent amino acids by acid hydrolysis.

3. Suggestion has been given that these amino acids may exist as

peptides in combination with the nucleic acids.

RFFERENCES

(*1*) Sevag, M.G, *Biochem. Z.*, **273**, 419 (1934)
(*2*) Holdsworth, E.S., *Biochem. J.*, **59**, 340 (1955)
(*3*) Yokoi, H., and Akashi, S., *Nagoya Med.* J., **3**, 91 (1955)
(*4*) Fiske, C.H., and Subbarow, Y., *J. Biol. Chem.*, **73**, 627 (1925)
(*5*) Grassmann, W., Hanning, K., and Plöckl, M., *Z. physiol. Chem.*, **299**, 258 (1955)

The Journal of Biochemistry, Vol. 45, No. 11, 1958

STUDIES ON THE RIBONUCLEOPROTEIN PARTIGLES

IV. SPONTANEOUS DEGRADATION AND RIBONUCLEASE ACTIVITY OF THE MICROSOMAL RIBONUCLEOPROTEIN PARTICLES

By YUTAKA TASHIRO

(*From the Department of Physiology, Faculty of Medicine Kyoto University, Kyoto*)

(Received for publication, June 9, 1958)

There have been some indications from the ultracentrifugal and turbidimetric studies of Petermann *et al.* (*1*, *2*) and Tso' *et al.* (*3*) that the microsomal ribonucleoprotein particles are very unstable and degrade spontaneously. It has remained quite vague, however, why these particles are unstable in themselves.

The authors (*4-6*) have reported that the microsomal ribonucleoprotein particles have ribonuclease activity, and the spontaneous degradation of these particles is probably attributed to this.

It is the purpose of the present communication to describe the ribonuclease and protease activities of the particles in more detail.

EXPERIMENTALS

Isolation and Purification of the Ribonucleoprotein Particles—The particles were extracted from rat liver microsomes with sodium deoxycholate and further purified by precipitation at pH 4.2 or by repeated centrifugation (*7*, *8*).

Substrate—Yeast ribonucleic acid, generously supplied by Dr. Y. Kuroyuwa of the Kirin Beer Research Institute, Amagasaki, was purified by glacial acetic acid in order to eliminate inhibitory action of the oligonucleotide on the ribonuclease activity (*9*). The pH was corrected to 7.0 with *N*/10 NaOH, and the final concentration was adjusted to 0.25 per cent. The autodegradation of ribonucleic acid was hardly detected when the solution was cautiously prepared before use.

Hammerstein Casein, Merck and Company, Inc., was dissolved in the same buffer, heated for 15 minutes at 100° and the pH was corrected to 7.0 (*10*).

The Measurement of Ribonuclease Activity—To 0.5-1.0 ml. of ribonucleoprotein solution (0.2-0.3 per cent), an aliquot of 0.25 per cent ribonucleic acid was added and incubated at 37°. At every given interval, samples were taken and an aliquot of ice cold *N* perchloric acid was added and the acid insoluble ribonucleic acid was determined by the Schmidt-Thannhauser's method slightly modified by Littlefield *et al.* (*7*).

The Measurement of Protease Activity—Proteinase activity was determined by the casein digestion method of Kunitz (*10*). The existence of nucleic acid in the reaction mixture

forced us to determine the protein content by Folin's reaction (*24*) or by Gornall's method (*25*), instead of the measurement of ultraviolet absorption at 280 mμ. Peptidase activities such as glycylglycine or glycyl-leucine dipeptidase were assayed by the titration of liberated carboxyl groups, following Grassmann and Heyde (*11*).

RESULTS

Spontaneous Degradation of the Ribonucleoprotein Solution—The ribonucleoprotein solution, dissolved in the Miller-Golder's buffer or in 0.1 *M* trismaleate buffer of pH 7.0, was incubated without enzyme, and acid insoluble ribonucleic acid was determined at every 5 minutes as described above. Only a small amount of protein and a considerable amount of ribonucleic acid were degraded.

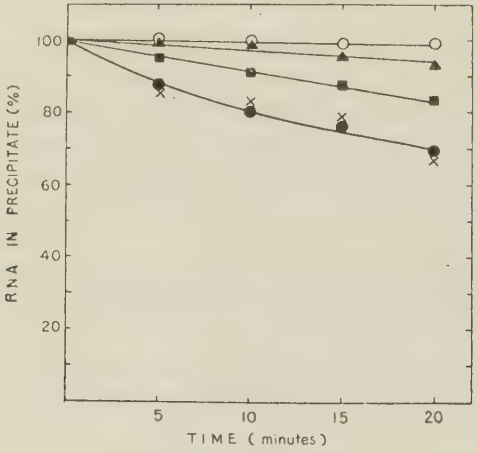


Fig. 1. Spontaneous degradation of the RNA of the ribonucleoprotein particles in the buffer at 23° (▲), 30° (■), and 37° (●), and in 0.25 *M* sucrose at 37° (×). Hardly any ribonucleic acid of the microsomes was degraded in 0.25 *M* sucrose at 37° (○).

While the higher was the temperature of incubation, the more ribonucleic acid was degraded (Fig. 1). About 30 per cent of ribonucleic acid of the solution was degraded after incubation for 20 minutes at 37°. This is in remarkable contrast to the autodegradation of the microsomes, because hardly any nucleic acid was removed from the microsomes under the similar experimental conditions.

Either 0.25 *M* sucrose or 0.125 per cent casein had no effect upon the degradation curve of the particles as suggested by the studies of Petermann *et al.* (*1*, *2*).

Effect of pH upon the Autodegradation of the Ribonucleoprotein Particles—To 1 ml. of the ribonucleoprotein dissolved in 0.1 *M* NaCl solution, an aliquot of the Miller-Golder's buffer of various pH was added and pH of the solution was determined by a glasselectrode in the cold. After incubation

at 37° for 10 (○) or 20 minutes (●), N perchloric acid was added and ribonucleic acid in precipitate was estimated as usual. As shown in Fig. 2, the autodegradation of the ribonucleoprotein particles depends upon pH of the medium, and they were most unstable at about pH 8.0.

The ultracentrifugal analysis on the autodegradation of the ribonucleoprotein has been reported elsewhere (*8*).

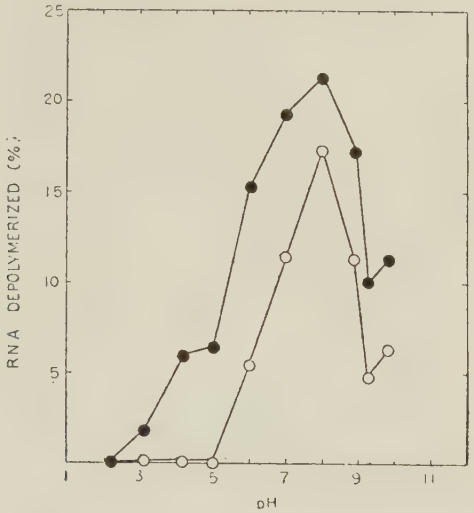


FIG. 2. Dependence of autodegradation of ribonucleoprotein-RNA upon the pH of the medium.

Ribonuclease Activity of the Ribonucleoprotein Particles—These results suggest that the ribonucleoprotein particles have ribonuclease activity in themselves. In order to confirm this, therefore, their ribonuclease activity was examined by incubating them with yeast ribonucleic acid. When 0.5 ml. of 0.25 per cent ribonucleic acid was incubated with 0.5 ml. of 0.2 per cent ribonucleoprotein at 37°, it was found that about 60 per cent of ribonucleic acid was depolymerized within 20 minutes as shown in Fig. 3.

Fig. 3 also illustrates the effect of temperature upon the nuclease activity of the particles, and the temperature coefficient Q_{10} between 30° and 37° was about 2.8.

As the pancreatic ribonuclease has been reported to be heat-resistant, the ribonucleoprotein solution was heated to 45–80° for 5 minutes, cooled to 0° and incubated for 20 minutes at 37°. Though the heating between 45–70° had hardly any influence upon the ribonuclease activity, a treatment at 80° for 5 minutes inactivates it nearly completely (see Fig. 4).

That the ribonuclease activity of the particles depends also upon pH of the medium, and their maximum activity was found at about pH 8.0 can be seen from Fig. 5. Roth (*12*) has demonstrated the presence of two ribonucleases in rat liver or kidney; the one is maximally active at pH 7.8–

8.0 (alkaline ribonuclease) and the second at pH 5.8–6.0 (acid ribonuclease). Fig. 5 suggests that the ribonuclease of the ribonucleoprotein is exclusively alkaline ribonuclease. The influence of NaCl on the ribonuclease activity

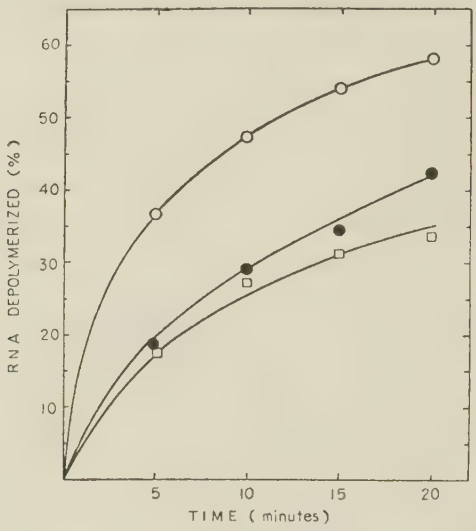


Fig. 3. Ribonuclease activity of the ribonucleoprotein particles at 23° (□), 30° (●) and 37° (○).

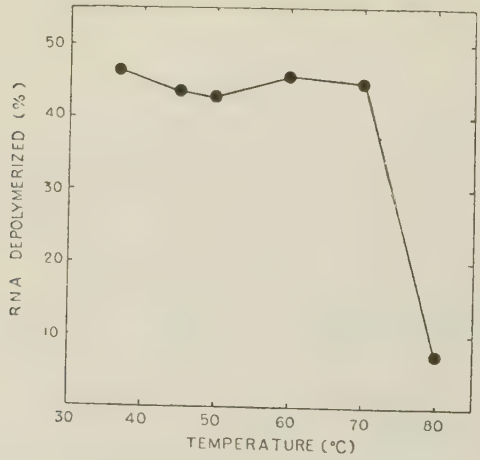


Fig. 4. Heat stability of the ribonuclease activity of the ribonucleoprotein particles.

was examined in the ionic strength range of 0.1, to 1.0. Higher activities were obtained at the ionic strength of 0.1 and 1.0, while the activity was minimum at 0.4~0.6.

Inhibitory Effect of the Supernatant upon the Ribonuclease Activity of the Ribonu-

cleoprotein Particles—Pirotte *et al.* (*14*) and Roth (*15*) have reported that the supernatant has an inhibitory effect upon ribonuclease activity. Therefore it was examined if the supernatant had similar inhibitory effect upon the ribonuclease activity of the ribonucleoprotein. To 0.25 ml. of 0.5 per cent ribonucleoprotein, 0.5 ml. of 0.25 per cent ribonucleic acid and 0.25 ml.

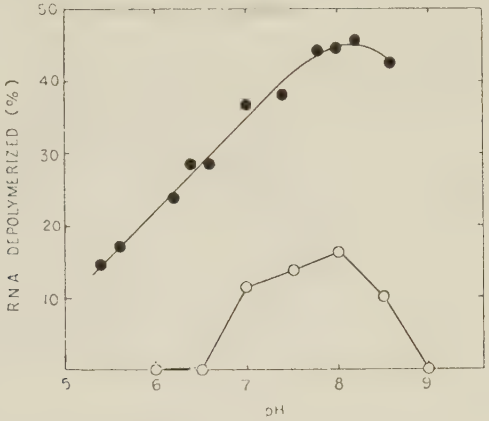


Fig. 5. Dependence of the ribonuclease activity of the ribonucleoprotein particles upon pH of the medium.

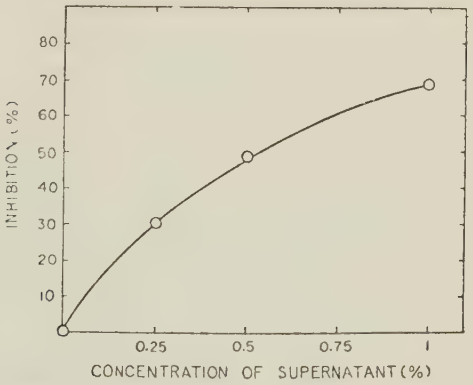


Fig. 6. Inhibition of the ribonuclease activity by the supernatant.

of various concentration of the rat liver supernatant obtained by the centrifugation at 105,000 × *g* for 120 minutes were added, and the inhibition was examined (see Fig. 6). As Petermann *et al.* (*1*, *2*) have reported that a dialysable material present in rat liver has the stabilizing action for the ribonucleoprotein particles, the supernatant was dialysed against 0.25 *M* sucrose for 48 hours in the cold, and the inhibitory effects of dialysed supernatant and dialysate were compared. Both the dialysed supernatant and dialysate showed inhibitory effect, though the former being considerably effective than the latter.

Comparison of the Ribonuclease Activity of the Microsomes with That of the Ribonucleoprotein Particles—As already stated, hardly any ribonucleic acid of the microsomes was degraded when the microsomes was incubated without added enzyme. This suggests a possibility that the ribonuclease of the microsomes is in a masked state and only manifests the activity when isolated as the ribonucleoprotein particles.

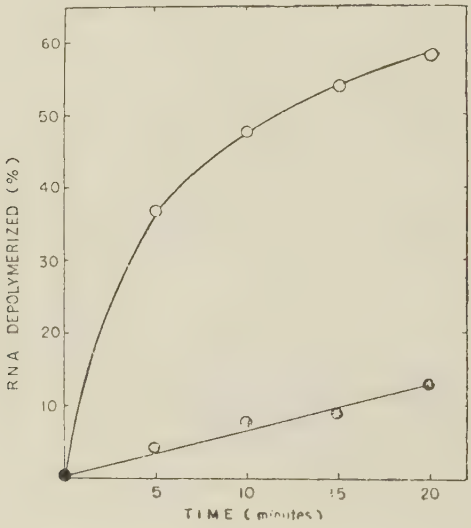


Fig. 7. Comparison of the ribonuclease activities and of the ribonucleoprotein.

The ribonuclease activity of the microsomes was compared with that of the ribonucleoprotein particles (Fig. 7). The ribonuclease activity of the microsomes containing as much ribonucleic acid as the ribonucleoprotein, was less than one tenth of that of the ribonucleoprotein, and accordingly the activity per unit weight of protein of the former was only about one fiftieth of the latter.

In view of the facts that 0.25 *N* H_2SO_4 can be used to extract total ribonuclease (*13*), and that the alkaline ribonuclease is reported to be resistant to 0.25 *N* H_2SO_4 (*15*), the microsome and ribonucleoprotein were treated with 0.25 *N* H_2SO_4, and the activites of these ribonucleases were compared. The former was enhanced about 50 x, and, that of the latter by about 1.5 x, while the total ribonuclease activity of the ribonucleoprotein per unit weight of protein was about twice stronger than that of the microsomes.

Protease Activity of the Ribonucleoprotein Particles—Protease and dipeptidase activities (glycylglycine and glycyl-leucyne dipeptidase) of the ribonucleoprotein were determined by the methods due to Kunitz and Grassmann respectively. The ribonucleoprotein indicated no such activity.

DISCUSSION

From the present findings it is highly probable that the ribonucleoprotein isolated from the rat liver microsomes has strong ribonuclease activity. Some considerations are, however, necessary before one can conclude that the ribonucleoprotein particles have ribonuclease activity in themselves; in other words, there is a possibility that the ribonuclease activity of the ribonucleoprotein may be due to a small amount of lipoprotein contamination, or that the ribonuclease which had been linked with the microsomes was released from it by the treatment with deoxycholate, thus contaminating the ribonucleoprotein particles during the isolation procedures. The former possibility can be excluded, becaase (*1*) the ribonucleoprotein purified by electrophoretic method shows similar ribonuclease activity, and because (*2*) the ribonuclease activity per unit weight of protein of the ribonucleoprotein was stronger than that of the deoxycholate soluble fraction (lipoprotein).

The elimination of the latter possibility is not an easy one, because surface active agents such as Triton x-100 are reported to release ribonuclease from cytoplasmic granules (*16*) and it is very likely that the acidic ribonucleoprotein combines *in vitro* with basic ribonuclease by ionic force. However as ribonucleoprotein seems to be unstable irrespective of the sources from which they were isolated, or of the method of isolation (*1*, *3*, *17*, *18*), it may be very likely that the ribonucleoprotein particles contain ribonuclease as a constituent. In this connection Elson's (*19*) recent finding of the latent ribonuclease activity of a ribonucleoprotein isolated from *Escherichia coli*, shall be consulted.

It is to be noted, however, that only a small part of total ribonuclease activity is found in the ribonucleoprotein particles. The mitochondria, although containing only a small fraction of the ribonucleic acid of the cell, contain a large portion of the ribonuclease activity (*20*, *13*). de Duve (*16*) has further reported that the ribonuclease of the mitochondria is exclusively localized in the so-called lysosome. The soluble portion of the cell also contains a considerable amount of alkaline ribonuclease in an inactive form (*13*).

It may be curious at a first sight that the ribonucleoprotein particles have the ribonuclease activity in themselves, but it is not improbable that their activity as a depolymerase is inhibited completely in the cell by the combination of the particles with the microsomes and by the inhibitory components contained in the supernatant.

Biological significance of the ribonuclease activity of the particles is quite vague. It may be responsible for the maintanance of the mononucleotide pool of the cell or for some synthetic reactions in the cell (*21*). Moreover, importance of the ribonucleoprotein for the incorporation of amino acids to protein (*7*), the existence of nucleic acid-amino acid complex in the pH 5 enzymes (*22*), and the structural studies on the ribonucleoprotein particles (*23*, *5*) strongly suggest that the ribonucleic acid is acting not as a static template but as an acceptor or a carrier of amino acids, and that the ribonuclease of the ribonucleoprotein is playing an important role of this transport mechanism.

SUMMARY

From the rat liver microsomes, ribonucleoprotein particles were isolated and the spontaneous degradation, ribonuclease and protease activities of these particles were examined.

1. Degradation of the nucleic acid seems to be mainly responsible for the autodegradation of these particles. The process is dependent upon temperature and pH of the medium, and the particles show the maximum unstability at pH of about 8.0.

2. The isolated ribonucleoprotein particles have the ribonuclease activity. Yeast ribonucleic acid was incubated with the ribonucleoprotein and it was found that the ribonucleic acid was easily degraded by the latter. Effect of various environmental conditions such as ionic strength, pH, and temperature upon the ribonuclease was examined and it was concluded that this ribonuclease was probably alkaline ribonuclease described by Roth. The supernatant solution had inhibitory effect upon the ribonuclease activity.

3. Free and total ribonuclease activities of the microsomes were compared with those of the ribonucleoprotein particles. The former contained almost all the ribonuclease in an inactive state, while the latter contained in an active state.

4. The ribonucleoprotein particles contained hardly any protease or dipeptidase activity.

5. Physical state of the ribonuclease of the ribonucleoprotein particles and its biological significance were briefly discussed.

The author wishes to express his thanks to Prof. K. Sasagawa and to Prof. A. Inouye for their encouragement, to Dr. K. Iwai for permitting us to use a Spinco Model L Ultracentrifuge throughout his experiments.

REFERENCES

(*1*) Petermann, M.L., and Hammilton, M.G., *J. Biophys. Biochem. Cytol.*, **1**, 469 (1955)
(*2*) Petermann, M.L., and Hammilton, M.C., *J. Biol. Chem.*, **224**, 725 (1957)
(*3*) Tso', P.O.P., Bonner, J., and Vinograd, J., *J. Biophys. Biochem. Cytol.*, **2**, 451 (1956)
(*4*) Tashiro, Y., and Sato, A., *Kagaku*, **27**, 367 (1957)
(*5*) Tashiro, Y., Sato, A., Takai, K., and Imai, Y., *J. Jap. Biochem. Soc.*, **2**, 615 (1957)
(*6*) Tashiro, Y., Ogura, M., Sato, A., Shinagawa, Y., Imai, Y., Hirakawa, K., and Hirano, S., *Proc. Intern. Symp. Enz. Chem.*, Tokyo and Kyoto, p. 436 (1957)
(*7*) Littlefield, J.W., Keller, E.B., Gross, J., and Zemecnik, P.C., *J. Biol. Chem.*, **217**, 111 (1955)
(*8*) Tashiro, Y., *J. Biochem.*, **45**, 811 (1958)
(*9*) Zittle, C.A., *J. Biol. Chem.*, **160**, 527 (1945)
(*10*) Kunitz, M., *J. Gen. Physiol.*, **30**, 291 (1947)
(*11*) Smith, E., Methods in Enzymology, edited by Colowick, S.P., and Koplan, N.O., Acad. Press, New York, Vol. **2**, p. 93 (1955)

(*12*) Roth, J.S., *J. Biol. Chem.*, **208**, 181 (1354)
(*13*) Pirotte, M.L., and Desreux, V., *Bull. Soc. Chem. Belg.*, **61**, 127 (1952)
(*14*) Roth, J.S., *Biochim. et Biophys. Acta*, **21**, 34 (1956)
(*15*) Roth, J.S., *J. Biol. Chem.*, **227**, 591 (1957)
(*16*) de Duve, C., Pressman, B.C., Gianetto, R., Wattiaux, R., and Appelmans, F., *Biochem. J.*, **60**, 605 (1955)
(*17*) Pirie, N.W., *Biochem. J.*, **47**, 614 (1950)
(*18*) Chao, F.C., and Schachman, H.K., *Arch. Biochem. Biophys.*, **61**, 220 (1956)
(*19*) Elson, D., *Biochim. et Biophys. Acta*, **27**, 216 (1958)
(*20*) Schneider, W.C., and Hogeboom, G.H., *J. Biol. Chem.*, **198**, 155 (1952)
(*21*) Schmidt, G., The Nucleic Acids, ed. by Chargaff, E., and Davidson, J.N., Academic Press Inc., Publishers, New York, Vol. **1**, p. 555 (1955)
(*22*) Hoagland, M.B., Zamecnik, P.C., and Stephenson, M.L., *Biochem. et Biophys. Acta*, **2**, 215 (1957)
(*23*) Tashiro, Y., Sato, A., and Furuta, Y., *Cytologia*, **22**, 136 (1957)
(*24*) Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., *J. Biol. Chem.*, **193**, 265 (1951)
(*25*) Gornall, A.G., Bardawill, C.J., and David, M.M., *J. Biol. Chem.*, **177**, 751 (1949)

The Journal of Biochemistry, Vol. 45, No. 11, 1958

IMMUNOCHEMICAL STUDIES ON INSULIN CONCERNING NEUTRALIZING ANTIBODIES

Using the Dale technique, Lewis (*1*) observed local anaphylactic contraction of uterine strips from previously sensitized animals with insulin. Anaphylaxis in guinea pigs sensitized and challenged with various insulin preparations were reported by Bernstein *et al.* (*2*) and Wasserman *et al.* *3*).

Lowell (*4*), Berson (*5*), Sehon (*6*) and others suggested that the development of resistance to insulin in patients following a course of insulin treatment was considered to be accounted for neutralizing factor against insulin in blood sera of patients.

There is, however, the opinion that insulin is not an antigen.

Moloney and Coval (*7*) reported that insulin can exercise a true antigenic effect as manifested by anaphylxis and by the production of neutralizing antibodies. Following thire technique, in our laboratory, it was evident that immune sera prepared according to their method had low activity.

In the present paper the conditions for the formation of more potent neutralizing antibodies against insulin have been studied in more detail.

The insulin preparation for immunization was made as follows. Ten ml. of a solution of insulin (Novo's Ultra Lente Insulin 40 units/ml.), pH 3.0 with HCl and containing phenol (1 per cent) was added to the mixture of 20 ml. of a paraffin oil and 10 g. of anhydrous lanolin and 100 mg. of heat killed human type tubercle bacilli (Aoyama B strain). Emulsification was effected by rapid mechanical stirring in a mortar.

Each guinea pig was injected subcutaneously by 2 ml. of antigen preparation into each of two separated areas. Thus a total dose of 0.85 mg. (20 units) of crystalline insulin was injected. This dose was repeated four weeks later and at other intervals as needed.

Blood samples were taken by cardiac puncture at interval of two weeks after the first inoculation, and the neutralizing activity of serum was tested by mouse convulsion assay procedure. Tests were carried out by injecting starved mice (pure strain: ddN strain, body weight: 15~20 g.) with serum with 0.25 units of insulin which, when injected in buffered saline or normal serum, caused convulsions in all of the mice.

The results are shown in Table I and indicate that 1 ml. of immune serum, which was obtained by the injection of antigen with added heat killed tubercle bacilli, can neutralize about 3~6 units of insulin 6 weeks after the first injection. The most potent sera which are obtained have shown a neutralizing activity of 25 units of insulin/ml.

In contrast to these results, it is shown that immune sera obtained by

TABLE I

Neutralizing Activity of Immune Serum

Composition of antigen	Guinea pig No.	Weeks after the first inoculation		
		2	4	6
		(*units/ml.*)	(*units/ml.*)	(*units/ml.*)
Insulin in adjuvant with heat-killed tubercle bacilli	1	1–2	4–8	6–8
	2	0.25–0.5	1–2	3–4
	3	0.5 –1	2–4	6–8
	4	0.5 –1	0.5–1	1–2
	5	1 –2	1–2	4–6
	6	0.5 –1	2–4	2–4
	7	0.25–0.5	2–4	1–4
	8	0.5 –1	2–4	1–2
Insulin in adjuvant without heat-killed tubercle bacilli	9	0–0.25	0–0.25	1–1.5
	10	0	0	0.5–1
	11	0	0	0
	12	0–0.25	0	0.25–0.5

Activity of immune serum was expressed with insulin units which was neutralized by 1 ml. of serum. No. 6, 7, 8 guinea pigs received a single injection.

TABLE II

Fractionation by Ammonium Sulphate

Saturation of ammonium sulphate	Neutralizing activity
0–33 %	+
33–50 %	+
50–66 %	–
66–100%	–

the injection of antigen without killed tubercle bacilli, have remarkably low neutralizing activity of 0.5~0.75 units of insulin/ml.

The immune serum (2 units/ml. neutralizing activity) was fractionated with ammonium sulphate. As shown in Table II, the neutralizing activity was present in the fractions precipitated by the half saturation of ammonium sulphate. The activity was not lost by dialysis.

Studies of the production of diabetes by the immune sera are under investigation.

We acknowledge with thanks Lente-Insulin given by Dr. Y. Nojima of the Kodama Shoji Co.

REFERENCES

(*1*) Lewis, J.H., *J. Am. Med. Assoc.*, **108**, 1336 (1937)

(*2*) Bernstein, C., jun., Kirsner, J.B., and Turner, W.J. *J. Lab. Clin. Med.*, **23**, 938 (1938)

(*3*) Wasserman, P., and Mirsky, I.A., *Endocrinology*, **31**, 115 (1942)

(4) Lowell, F.C., *J. Clin. Invest.*, **23**, 225 (1944); **23**, 233 (1944); **26**, 57 (1947)
(5) Berson, S.A., Yalow, R.S., Bauman, A., Rothschild, M.A., and Newerly, K., *J. Clin. Invest.*, **35**, 170 (1956)
(6) Sehon, R.H., Kaye, M., McGarry, E., and Rose, B., *J. Lab. Clin. Med.*, **45**, 765
(7) Moloney, P.J., and Coval, M., *Biochem. J.*, **59**, 179 (1955)

Department of Medical Chemistry,
Faculty of Medicine, Kyushu University,
Fukuoka

YUICHI YAMAMURA
MASAYASU KITAGAWA
KAORU ONOUE

(Received for publication, August 20, 1958)